The Molecular Biology of Epidermal Growth Factor Receptor and its Role in Human Breast Cancer.

A Thesis Submitted for the Degree of Master of Science in the Department of Biochemistry of the University of Glasgow.

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ABSTRACT.

This thesis deals with a number of aspects of Epidermal Growth Factor Receptor (EGF-R) molecular biology and also EGF-R's involvement in breast cancer.

The current literature on the biology of breast cancer and on the structure of both the EGF-R gene and protein are reviewed, as are the ways in which advances in technology have contributed to our knowledge in these areas.

The link between EGF-R structure and function is emphasised and this leads into a discussion on EGF-R signal transduction. EGF-R is a 160 K.D. polypeptide which has intrinsic tyrosine kinase activity. The cellular substrates of the kinase are largely unknown but many of those that have been identified have been shown to be important structural and regulatory proteins.

EGF-R also has autophosphorylation ability i.e. EGF-R phosphorylates itself as well as exogenous substrates. This is a very important process and is believed to have a regulatory function, possibly mediated by structural features of the receptor and is discussed in some depth.

In order to more clearly understand the ways in which EGF-R may be involved in human carcinogenesis, and to put the problem in context, a review of the very large and complicated area of cancer biology is included. In addition, the specific case of breast cancer molecular biology is examined in some detail.

EGF-R has been shown to be an important regulator of growth in both normal and cancerous cells. In breast cancer, EGF-R has been reported to be an index of poor prognosis, both in relation to disease free interval and total survival.

The current literature concerning EGF-R's role in human breast cancer development is often conflicting. Points reviewed in this thesis include the autocrine theory of growth factor involvement in growth regulation, EGF-R and breast cancer cell proliferation, and EGF-R as a potential therapuetic target.

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List of Abbreviations.

The Amino Acids.

A	Ala	Alanine
B	Asx	Asparigine/Aspatic Acid
C	Cys	Cysteine
D	Asp	Aspartic Acid
E	Glu	Glutamic Acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
Z	Glx	Glutamine/Glutamic Acid

ATP: Adenosine Triphosphate

DAG:sn-1,2-Diacylglycerol

EGF:Epidermal Growth Factor

EGF-R:Epidermal Growth Factor Receptor

ER:Oestrogen Receptor

FGF:Fibroblast Growth Factor

H:Hydrogen

Ins:Inositol

IGF:Insulin Like Growth Factor (Types I and II)

Na:Sodium

NGF:Nerve Growth Factor

PA:Plasminogen Activator

PDGF:Platelet Derived Growth Factor

PDGF-R:Platelet Derived Growth Factor Receptor

TGF-alpha:Transforming Growth Factor-alpha.

TGB-beta:Transforming Growth Factor-beta.

Chapter One: INTRODUCTION.

1.Introduction.

Cancer is a disease that has been known since antiquity. As will be described later, despite this and our rapidly accumulating bio-medical knowledge, little advance has been made in the treatment of solid cancers. Nowhere is this more true than in the specific example of breast cancer. This form of cancer kills many hundreds of thousands world wide per year.

For this reason, research into the causes and molecular mechanisms of breast cancer is a very large and active area encompassing disciplines such as cell biology, immunology, genetics and biochemistry. It is hoped that such an approach:with a number of different, but complementary avenues being explored simultaneously will provide some answers to the many questions that remain about the natural history of tumours.

It is also anticipated that such basic and applied research programmes will allow new drugs and other therapeutic regimes to be designed and so facilitate patient management.

This thesis will examine the role of only one cellular protein in the genesis of one specific human cancer. The protein is the Epidermal Growth Factor Receptor or EGF-R and the cancer is breast cancer.

EGF-R is, as its name would imply, the receptor for the growth factor Epidermal Growth Factor (EGF). It is held that the binding of ligand to EGF-R begins a cascade of actions which eventually culminates in the expression of certain cell proliferation regulation genes.

In normal circumstances, this signalling pathway is stimulated such that the processes of cell death and replacement are balanced. The theories state that in some way, for example by alteration of the structure of the EGF-R itself, this balance is disrupted as occurs in cell transformation, and cell proliferation occurs in an uncontrolled (or less controlled) way. The result is the production of a unruly mass of cells-clinically diagnosable as a tumour.

The examination of growth factors and their receptors-of which EGF-R is an example has proven to be a very profitable route to take. Since the initial discovery of growth factors in the nineteen-sixties their importance in cellular growth and its control has been beyond dispute. Their use, coupled with cell culture systems has meant that a controlled, manipulable experimental environment can be set up to study the cause-and-effect relationship between growth factors and cell division. Today, modern techniques such as reverse genetics, DNA sequencing, gene alteration and cloning have advanced and refined such studies.

It was studies using some of these techniques which demonstrated that EGF-R was involved in the process of breast cancer development. In the first instance the presence of EGF-R was detected, then theories as to the molecular mechanisms involved were able to be adumbrated. Today, it seems likely that EGF-R could be an important target for therapies against many forms of cancer, not just breast cancer.

Much data exist on many aspects of EGF-R and its relationship to cancer. The intention of this thesis is to bring together and review certain areas of particular importance.

To set the scene, the biology of cancer will be discussed. A consideration of questions such as what is cancer?, how is cancer caused and an appreciation of the history of the problem and the various models of carcinogenesis will allow the problem of breast cancer to be put in context.

Breast cancer itself will then be discussed. A cellular biological and molecular genetical approach is taken here since I feel that these are the two more important areas in terms of contribution to and the advancement of knowledge and potential for the future.

Next is an analysis of the molecular biology of EGF-R. Many experiments have pointed to the crucial nature of the actual structure of the receptor to its functioning. Questions of structure at both DNA and protein levels and related aspects are reviewed.

Much weight in this thesis is given to EGF-R signal transduction. The reason for this is that it is believed that here lie the ultimate answers to questions about EGF-R and its role as a growth factor and in carcinogenesis. Briefly then, EGF-R's function as a protein kinase (tyrosine, serine or threonine) is reviewed along with EGF-R's effects upon the inositol lipid metabolism and ionic fluxes of a cell and the cotribution of G-proteins to these processes.

With sufficient background information having been discussed, the role of EGF-R in breast cancer is discussed. A number of points are raised, such as EGF-R and cell transformation, tumour size, stage, grade and sub-groups. Looking forward is a section on how EGF-R might be useful in terms of breast cancer therapy.

Chapter Two:
CANCER BIOLOGY.

2.1.Introduction.

A tumour is a contiguous mass of cells derived from a progenitor cell which in some way has lost it's ability to respond to the normal cellular growth control mechanisms.

Tumours can be of two types. Firstly there are benign tumours which are encapsulated and cannnot invade the tissue which surrounds them. The second group of tumours are the malignant tumours. These cancers are marked by their ability to invade the surrounding tissue and spread to other sites in the body, a process known as metastasis. These two properties are responsible for the lethal nature of malignant tumours.

Malignant tumours can be Carcinomas (derived from epithelial cells); Sarcomas (arise in the supporting tissues of the body) or Leukaemias/Lymphomas (which arise in the blood forming cells in the bone marrow and lymph nodes).

In addition, the precise definition of a malignant tumour depends on the organ in which it arose and upon the type of cell involved.In humans approximately 100 different types of malgnant tumours are known (Franks and Teich, 1986). However, cancers of three main organs are responsible for around half of all cancer deaths: those of the lung, the large intestine and in females, the breast.

Cancer may affect all multicellular organisms. Evidence has shown that alterations in cell growth and development and in the control of these processes lie at the centre of this disease. Knowledge of the processes underlying cell growth and differentiation will be of great importance to an understanding of malignancy.

Cancer is a disease that has been known since antiquity (see Franks and Teich,1986). In recent times, though, the incidence of cancer has been increasing rapidly-and a notable acceleration has taken place this century. In 1986 it was estimated that some 20% of all those presently alive would die from some form of cancer (Franks and Teich,1986).

This increase in the incidence of cancer might be explained by the fact that advances in medicine (in the Western world at least) coupled with improvement in public health has meant the people are living longer today. These breakthroughs have virtually eradicated the threat to health from infectious diseases which at one time were the major killers. The main beneficiaries have proven to be the most vulnerable in our society: the young and the old.

The increase in life span has caused a change in the age structure of the population that has been observed. This means

that cancer: which is mainly a disease of the elderly, is accounting for proportionally more deaths.

Other contibutary factors to the increase in cancer deaths is the fact that the general public are more aware of the dangers and the warning signs of cancer. This, coupled with numerous screening programmes means that in modern times more people are making themselves available to diagnosis, so more cases are being reported. As advances are made in the laboratory better diagnostic tools come into use, and consequently, cancers are being diagnosed at a controllable stage. Allied to this is the fact that improved therapeutic regimes mean that at least for some cancers, there is now a better chance of survival once a tumour has been diagnosed.

It should be noted that these increases in cancer incidence are occuring in spite of the vast number of medical advances that have been made. It seems that science has made little impact on the diseases of the aged-possibly because little of their underlying molecular nauture is known. The example of breast cancer is particularly illustrative of this point.

Although diseases of the heart and vascular system will kill more people than will cancers (Franks and Teich,1986), cancer is much more feared. Many will develop cancer of some

form at some stage in their lives but a great many more people will live in fear of the disease.

2.2 Causes of Cancer.

Apart from the possible exception of cigarette smoking, the causes of human cancer remain largely obscure. The link between age and deaths from cancer suggests that prolonged exposure to certain agents (endogenous and in the environment)are involved in the induction and/or progression of tumour growth. The lapse in time from exposure to presentation with clinical symptoms and the fact that the aged population is the one which most commonly developes this condition implies that a considerable period of time (i.e. either long exposure to a single agent or a number of separate insults)is required for carcinogenesis to be induced and/or proceed.

Among the endogenous causes of cancer can be counted various genetic predisposing states, for example mutations at the rb and p53 loci (reviewed in Glover and Harris, 1989, Finlay et.al.,1989 and Vogelstein and Kinzer, 1992), and also conditions such as Xeroderma Pigmentosa (Darnell et.al., 1986).

The group of exogenous causative factors is very large indeed ranging from physical agents, for example, X-ray, ionising

radiation and UV light to organic chemicals such as aromatic amines, creosote, mineral oils, soot, tobacco smoke, tar-pitch, polycyclic hydrocarbons, azo dyes, oestrogens and alkylating agents. In this grouping are also inorganic chemicals: asbestos, arsenic, chromium and benzene.

Viruses for example, Epstein-Barr virus, PAPOVA viruses and HTLV virus have for many years been associated with tumours. This area is outwith the field of this thesis but is reviewed in (Varmus, 1983).

Dietary factors, in particular a diet rich in animal fats, low amounts in fibre and high in animal protein is thought to contribute to the genesis of certain cancers-in particular, colon cancer. Alcohol and general over nutrition as well as the presence of a variety of preservatives and additives in food may also be involved in this process.

Finally, some miscellaneous factors and agents, such as repeated mechanical injury or abrasion, sexual promiscuity (see viruses above), the presence of particular parasites: Schistosoma hematobium and Chlonorchis sinensis (Franks and Teich, 1986) and the use of immuno-suppressive drugs are also associated with tumours and/or the process of tumour development.

2.3.Epidemiology of Cancer.

Epidemiology is the study of patterns of a disease (not necessarily cancer) in a population, with the ultimate aim being to eradicate/ameliorate the effects of the etiological agent(s) thus identified and so eradicate or control the disease. Epidemiology is statistical, with the conclusions drawn being expressed as probabilities.

Descriptive epidemiology identifies susceptible groups, for instance in terms of age, location or racial background. Such studies into breast cancer incidence have shown that diet has a large role to play in the development of this disease. Japan has a low rate of breast cancer, but immigrants from Japan to the U.S.A. where incidence is high, have, within one or two generations a breast cancer incidence rate which is the same as the host nation (Franks and Teich, 1986). This is because of the adoption by the immigrants of the local culture and lifestyle.

Such studies-which have often been repeated, show that the environment does exercise a considerable influence over the deveplopment and evolution of cancers in humans. The level of civilization/development of the society is often positively correlated with tumour incidence, as revealed by the link between refined foods and fatty diet with cancer of the colon, and cigarette

smoking with the incidence of cancers of the lung.

Host factors are important too. One of the most significant of these factors is the role of the immune system in carcinogenesis (This topic is outwith the scope of this thesis but is reviewed in Rygaard and Povlsen, 1976, Young and Elliott, 1989 and Johnson et.al., 1989(a)).

The one common factor for that all the agents listed above and identified by epidemiological studies is that they all, either directy or indirectly, damage DNA or affect gene expression. Thus, while many cellular changes have been demonstrated to occur as a normal cell becomes cancerous, it would seem that at the most elementary level, cancer is a genetic disease (discussed in section(2.8)).

2.4. Models of Carcinogenesis.

A.Hippocrates.

In the fourth century B.C. Hippocrates named the spreading, deadly tumour that he often saw Carcinoma, and chronic ulcerations-including benign tumours Carcinos (De Moulin, 1989). Carcinomas were thought to depend on an excess of black bile. Even at this stage, the lack of an effective treatment for carcinomas especially those that were well advanced or

metastasised was evident since Hippocrates and his followers did not even attempt to treat such cases. He noted people often survived longer without intervention, and given the lack of sterile surgery or anaesthetic, this course of action was probably very wise (see also section (6.9) for specific details on breast cancer). Such an approach still has value today.

B-Galen.

Practicing in the century following Hippocrates, Galen catergorised tumours in a very insightful way, defining "tumours according to nature" (such a the natural growth of the body); "tumours exceeding nature" (such as the healing of broken bones) and "tumours contrary to nature" (for example neoplasia) (De Moulin, 1989). Galen had therefore distinguished between normal hyperproliferative states and diseases where proliferative control had been lost. Galen also described the similarity between the lesions caused by a tumour and the shape of the crab.

C.The Cellular Theories of the Nineteenth Century.

Early in the nineteenth century, Johannes Mueller showed a tumour was composed of a number of cells (Franks and Teich,1986). This led to research to identify the difference

between normal and cancerous cells with the underlying hypothesis that cancer was a disorder of cells.

Those working in the field were split into two main camps: those who though that normal cells were in some way converted to cancerous cells (such as Muller) and those who felt that cancer existed within the cancerous cell right from the creation of that cell and that this phenotype was only expressed later (such as Leannec). The later point of view was supported by the findings of Pasteur as illustrated by his Germ Theory.

Modern science has shown that elements of both theories are accurate:the mutation theory (see section 6.9) being in line with (1) and the existence of anti-oncogenes and genomic imprinting (see Section 6.9)) supporting (2).

Parallel with these studies were experiments such as those in tumour transplantation and cell culturing. These were important not only in allowing certain features of carcinogenesis to be characterized but also as means of facilitating the in vitro analysis of the disease.

D. Twentieth Century Theories.

Building on the advances of previous years and using new techniques such as microscopy, theories as to how tumours were initiated and how they progress were proposed.

Theodore Boveri in 1914 outlined his Somatic Mutaion Theory. This theory was based on two major observations: that when studied under the microscope, cancer cells often have chromosomal abnormalities. Secondly, the genomic alterations underlying carcinogenesis are heritable since cancer cells do not naturally revert to the normal state (Friedberg, 1986).

Viruses have also been implicated as causes of cancer. This subject was mentioned previously (see section 2.2.), but briefly, in chicks, cats and mice, certain leukemia viruses can cause cancers to develop. Such viruses can be spread vertically as well as horizontally and it is likely that the immune system is intimately involved in virally induced carcinogenesis (that is to say a subversion of the immune system).

Cases of human childhood leukemia often arise in clusters. Originally viruses were suspected (reviewed in Galloway, 1990) but now it is thought that physical causes such as ionising radiation are more likely to be to blame. (See the following for a review of this area concentrating on the recent and current controversies surrounding nuclear power stations: Gardner et.al., 1990 (a) and (b) and Evans, 1990).

Some human tumours have been found to actually contain parts of viral genomes, for example in lymphatic cancers

and Hodgkin's Disease; Epsein-Barr virus (reviewed in Young et.al., 1989 and Dawson et.al., 1990) and human Papilloma viruses in cervical cancer (reviewed in Martin et.al., 1989; Durst et.al., 1983; Androphy et.al., 1985; Schwartz et.al., 1985; Gius and Laimins, 1989 and Phelps et.al., 1988).

A final point to note is that study of certain viruses (in particular retroviruses) has provided much insight into the molecular mechanisms of carcinogenesis. The most noteable of these achievements is the identification and eventual characterization of ONCOGENES (See section 2.8.).

The multi-step theory of carcinogenesis is a genetic based theory stating that cell proliferation control depends upon a number of separate genetic controls, all of which have to be inactivated for a cancer to develop (Commings,1973). The chance of all the required changes happening increases in direct proportion to age. Evidence for this is provided by the previously outlined details on the age distribution of cancer statistics. Risk of cancer inceases as the nth power of age (Cairns, 1986). Plotting of the logarithm of incidence against the logarithm of age generates a slope which reveals the number of mutation events needed to create a cancer. For most cancers, five mutations are required (Cairns, 1986).

Three phases must be gone through in order for a tumour to be clinically identifiable. Firstly there is *Initiation*. Initiation involves a change in the proliferative ability of the cell perhaps due to certain agents interacting with the DNA. Normal control of cell proliferation is lost and this imparts upon the cell an evolutionary advantage since it is more able to divide and so replicate itself. In this way, the altered cell can overgrow the neighbouring cells.

Cell proliferation is vital to initiation and it is thought that it may act to fix the geneomic change into the DNA in a way which may be related to the DNA repair mechanisms (Cailleau et.al.,1974). Examples of initiating agents are chemical carcinogens, viruses, ionising radiation, UV light and DNA replication errors.

Promotion is the phase which follows initiation after a latent period which is usually of significant duration. This step sees the expansion of this one progenitor cell to a population of cells i.e. clonal expansion (Klein and Klein, 1986).

In addition, it is thought that the promoting agent in some way(s) alters the microenvironmet of these altered cells so that clonal expansion is possible.

Promoting agents themselves do not cause cancer, they induce cells which are already in an initiated state to divide. To act then, such agents require cell growth and certain protein

factors. Promoting agents probably interfere with normal differentiation processes, and examples are inflammation and hormones.

Finally there is *Progression*. This is the advancing of the mass of cells to more and more malignant states in a step-wise process (Klein and Klien, 1986 and Foulds,1954). Again, the exact nature of the steps involved here is not known but it is likely that a number of separate genetic alterations are responsible; for example, mutations of oncogenes (see section 2.8.) and anti-oncogenes (see section 2.8.). There is also the role of a number of largely unidentified modulating agents which affect tumour spread such as expression of MHC antigens (Klein and Klein, 1986 and Dalianis et.al.,1979).

2.5.Tumour Development.

Once a cell has been initiated, a series of changes occurs, the rate of which depends on changes in the cell and changes in the host. That is to say that there is a degree of de-differentiation (or anaplasia)involved and normal characteristics are lost (Franks and Teich,1986). Such de-differentiation can be graded by a pathologist and there is a correlation between the grade of the tumour and the rate of tumour growth.

For example, a grade I tumour is the most slowly growing and most differentiated type of tumour. Grade IV tumours are very anaplastic and grow very rapidly. Grading often correlates with survival statistics too with better differentiaed tumours having a better prognosis (see section 6.8.).

Tumour grading describes the degree of differentiation of a tumour cell as measured cytologically and histologically. Tumour staging, on the other hand, is concerned with the amount of metastatic spread. Tumour grade and stage are not directly related and the growth rate of the tumour is not directly related to it's metastatic potential.

Tumours are not homogeneous masses even though they arise from a single progenitor cell (or a small number of cells in the case of polyclonal tumours). This is because clinically detectable tumours have usually been in existence for long periods of time. They have therefore undergone many rounds of cell division so that variation and selection will have occured resulting in different cellular populations being created.

The average tumour will therefore consist of a mixed population of cells differing in structure, function, growth potential, resistancies and ability to invade and metastasise.

2.6.Cancer Cell Biology.

2.6.1.General Details.

Cancerous cells can often be distinguished from normal cells by a number of cellular characteristics. Malignant cells are usually less well differentiated than their normal counterparts, as identified by alterations in cell surface antigens and the loss to varying degrees of cell specific functions.

Microscopically, cancerous cells have the following characteristics: a high nucleus-to-cytoplasm ratio; many and enlarged nucleoli; a large number of mitoses and a reduction in specialisations of intracellular structure (Darnell et.al., 1986). It should also be noted that these are in addition, characteristics of any rapidly growing cell. Some other characteristics are increased saturation density, loss of anchorage dependent growth, loss of Contact Inhibiton of Locomotion, altered cellular morphology, enhanced aggregation by lectins, reduction or total loss of surface fibronectin and the loss of microfilaments.

Some very important changes take place at the plasma membrane. For example, in normal cells, cell adhesion and a slowing down of amoeboid motions are usually observed upon contact with some outside surface: contact inhibition of locomotion. Soon after this, actin filaments appear from under the ruffled edges and these areas loose their morphological identity and

become indistinguishable from less active regions of the plasma membrane. In a cancer cell though, normal cellular affinities are lost and the cells cannot form tight adhesive junctions so can associate with virtually any other cell type. As a result, contact inhibition of locomotion is lost, and it is thought that this underlies the phenomenon of invasiveness.

2.6.2. Metastasis.

The ability to metastasise is the single most important feature of a maligant tumour because once a tumour has spread from it's original site and set up secondary tumours at distant locations, the chances of combating the cancer are very greatly reduced or even eliminated. If a tumour remained fixed in one site as a benign tumour does then it is likely that surgical intervention would be more successful.

Cancer may therefore be thought of as a condition characterized by the loss of positional determinants which, in turn, has it s basis at the genetic level. Thus, although various alterations of DNA are at they very centre of the problem, cancer can also be legitimately regarded and studied as a cellular biological problem. Many therapeutic, diagnostic and prognostic approaches such as those using monoclonal antibodies (See Drebin et.al.,, 1985; Masui et.al., 1984 and Hudziak et.al., 1989 (a) and

(b))and cytoskeletal disrupting agents (Darnell et.al., 1986) are underpinned by cell biology.

A number of separate stages can be identified in metastasis:

1-Separation of a tumour cell from the primary tumour mass.

2-Invasion of the subcellular matrix.

3-Penetration of the basement membrane of a blood vessel.

4-Survival by the cell of the journey through the vascular system:the fighting off of various agents of the immune sysytem.

5-Identification of a suitable secondary site and escape from the blood vessel.

6-Lodging at the new site and survival there.

7-Invasion of the substratum of the new site.

8-Inducion of angiogenesis (see below).

Cancer/normal cell hybrids were found to have normal phenotypes. This implies that the normal cell has certain factors which prevent the development of the cancerous state, i.e. growth was again brought under control (Thompson et.al., 1992). The most likely explanation is that metastasis in part at least depends upon the inactivation or the loss of inhibitory genes or "anti-oncogenes". This will be discussed in section 2.8.).

A gene: nm23 has recenty been identified which is thought to have anti-metastatic properties (Hennnessy

et.al.,1991). The gene is associated with good prognosis in breast cancer (Hennessy et.al.,1991)and reduced levels at the mRNA and protein levels are associated with enhanced metastatic potential (Bevilacqua et.al.,1989).

Metastasis is not a random process, since patterns of spread are noted. For example, neuroblastomas usually metastasise to the liver, osteosarcomas often spread to the lung and bresat cancers to the bones (Franks and Teich,1986).

This important observation has clinical significance yet it's biological basis is very mysterious. The following theories have been proposed. Selectivity may be due to blood flow in the secondary organ because the most commonly affected sites are those with good blood supplies. Alternatively, it may be that the original and secondary sites are similar in certain ways such as in terms of growth factor, hormone or immunological status.

2.6.3.Tumour Invasion.

The problem of how a tumour cell can either break into or out of a blood vessel and lodge in a secondary site is a crucial one. There are three likely ways for this to happen:firstly it could be the result of mechanical pressure due to the fact that the increased proliferation rate causing the tumour mass to rapidly

enlarge. Release of various lytic enzymes may also be responsible and is suported by the observation that the normal cells which surround a tumour are often badly damaged.

Tumours also have high levels of polymorphonuclear neutrophils and monocytes which have many degradative enzymes. The fact that tumour cells have increased motilty might also mean that they are more able to cross these barriers. Finally, a combination of all these hypotheses could be reposonsible for invasion by tumour cells, with a different modes being adopted in different tissues.

2.6.4.Angiogenesis.

To grow, a tumour cell must be supplied with an adequate vascular system to bring to it nutrients and remove from it various wastes and toxins. A tumour at the in situ stage, approximately one millimeter in diameter (see Franks and Teich, 1986) has no such blood supply and so it cannot expand in size and metastasise.

Growth therefore depends on tumour vascularisation or angiogenesis, and this is thought to be due to the release of Tumour Agiogenesis Factor (TAF) from tumour cells. TAF causes blood vessels to penetrate the tumour and allow it to grow rapidly with spread as an ultimate consequence.

Normal cells usually only express angiogenic molecules such as PDGF and TGF-alpha during embryogenesis, growth, development and wound repair (see Heldin et.al., 1985). This implies that angiogenesis is necessary for tumour growth, but it is not sufficient for this process to take place.

2.7.Cancer Genetics.

A.Introduction.

Although the environment exerts a considerable influence over the induction and progression of cancer, there is much evidence to support the point of view that cancer has a genetic basis:

1-Cancers are clonal in origin.

2-Genetic markers can be used to identify the clonal origin of cancers at the cellular level.

3-Cancers often have genomic alterations, and particular cancers often have specific chromosomal alterations.

4-Many carcinogens are also mutagens.

5-Defects in the ability to repair DNA increase the somatic mutation rate and the rate of cancer incidence too.

B.Classical Genetical Analysis of Cancer.

Scrutinization of family data has show that in some cases, inherited predisposition to cancer depends on a single

recessive or dominant Mendelian assorting factor. An example is that of Xeroderma Pigmentosa (XP). This is a recessive condition which is due to an inherited defect in the DNA repair system .

Such cases however, constitute only a small proportion of cancer cases. The fact that Ataxia Telangiectasia heterozygotes may have an increased susceptibility to develop a number of cancers may mean that such conditions have practical significance (Swift et.al.,1980).

Cases involving only one gene are probably in the minority. It is more likely that cancer is a mutifactorial condition-that is to say that it depends on a number of individual genes.

Multifactorial conditions are by their nature very difficult to analyse. The usual approach has been to study families in which the condition of interest commonly occurs.

This type of investigation is complicated by the fact that families also tend to share a common environment, as well as a genetic inheritance-that means they will have been exposed to the same range of enviromnental factors, viruses and other forms of carcinogens. The contribution of exogenous and endogenous factors therefore becomes hard to separate out from each other.

For these reasons, the incidence of cancers in twins is often studied-with monozygotic twins being the most informative

since they are genetically concordant. This genetic identity underlies the studies. The principle is that twins are usually exposed to a common environment, and so any differences in the incidence of disease is likely to be due to differences at the DNA level. Analysis of their DNA may lead to the identification of the actual areas of the genome which are the root cause of the condition-and maybe even the genes responsible for the phenotype.

Family studies are important too and have yielded significant information such as in the cases of colon carcinoma (Bussey et.al.,1978). The question of the general significance of these studies remains.

The usefulness of such studies is limited because all genes work in a background of other genes: this is the concept of Penetrance. This means that although a gene may have been identified in the laboratory as being involved in this process of carcinogenesis, whether it actually does so in vivo is unanswered. This matter is further complicated by the fact that in addition, different genes have different levels of penetrance and expressivity which will also affect how much they contribute to the phenotype.

C.Germ Line Mutations.

The importance of germ line mutations is that if it is held that carcinogenesis depends upon a number of mutational events, then if one of these required events has been inherited, all of the somatic cells will have this alteration too, i.e these cells aleady carry one of the genetic changes which is required for tumourigenesis (Knudson,1971). The individual who has inherited such an alteration will therefore be at increased risk of developing a tumour.

The Knudson theory is based on a model where two mutational steps are required to produce a tumour (Knudson, 1971, 1985 and 1986). This hypothesis is controversial since often more that one genomic change is needed to induce tumour growth. The idea of pre-existing alterations inherited via the germ line which causes a dominant inherited susceptibility to cancer does now have much supporting evidence and effectively explains the clustering of certain cancers in families and the inherited tissue susceptibility to cancer a seen in Retinoblastoma (for further details see Dryja et.al., 1989; Zhu et.al., 1989; Sapienza, 1991 and Weinberg, 1990).

Genomic alterations are obviously important in cancer development but these are not the only significant

alterations in this process. Changes in the pattern of gene expression are crucial too, but this need not depend on the physical alteration of DNA. Such epigenetic changes are also stably inherited at the cellular level and are difficult to separate from the effects due solely to mutation.

Data derived from studies on Retinoblastoma show the exact nature of the tumour and the timing of its occurence depends on whether one or both parent carry the appropriate gene (see the reviews cited above). The interpretation of such observations is that both genetic and epigenetic factors are involved in the formation of retinoblastoma. The genetic predisposition to cancer development is reviewed in Hansen and Cavenee, 1987 and 1988.

D.Chromosomes and Cancer.

Improvements in microscopy showed that most tumours have structural and/or numerical chromosomal alterations. Some of these changes are consistently associated with particular types of tumours. The locations of chromosomal rearrangements often identifies genes which are critically involved in carcinogenesis.

The first specific chromosomal abnormality observed in a human tumour was seen in Philadelphia in 1960 by Nowell and Hungerford (Nowell and Hungerford, 1960). They found that in the leukemic cells of patients with Chronic Myelogenous Leukaemia (CML)there was an abnormally small chromosome: the Philadelphia chromosome (Ph').

In the early 1970s chromosome banding techniques were coming into use. This, along with short term culturing methods to improve the yields of divding cells, allowed a more precise definition of the rearrangements involved in carcinogenesis and the identification of new ones.

There are a large number of different genomic alterations associated with cancers, a summary of the ones which have been extensively studied is listed below.

Table One: Chromosomal Alterations and Cancer.

Type of Alteration	Details	References
DNA Structre	Changes in tautomeric form of bases are common in response to mutagens; increased DNA supercoiling may facilitate integrative recombination.	Tse-Dihn et.al., 1984 and Wood and Lindahl, 1990.

DNA Repair	Is faulty in Xeroderma Pigmentosa and as a result UV induced DNA damage is not repaired.	
DNA Replication	Chromosomal non-disjunction is thought to underlie many of the genomic alterations seen in tumours. It has serious implications for the cell cycle and for proto-oncogene expression. Genomic destablisation is also associated with secondary changes which may allow tumour progression.	Holliday,1989; Fantes et.al., 1975; Klien , 1981 and Klein and Klein,1986.
Chromosome Structure.	Reduction in the size of Telomeres has been found to be involved in tumour development and also ageing. Telomere lengths reflect the mitotic history of cells because 4 base pairs are believed to be lost from the telomere per division cycle.	Jankovik et.al. 1991.
Genomic Translocations	Can affect the biochemical functions and expression of genes. The most studied example is that of c-myc and it's role in Burkitt's lymphoma. Fusion of c-abl and bcr is common in cases of Chronic Myelogenous Leukaemia which have a Philadelphia chromosome.	Croce and Nowell, 1986.

Amplifications	Common in cancers, especially the amplification of oncogenes. Oncogene amplification may contribute to oncogenicity. Somatic amplificaton of genes is an adaptive response to environmnental stress which is often manifest as Heavily Staining Regions and Double Minute Chromosomes.	Dalla-Favera et.al., 1982.
Genetic Transposition	Recombination allowing the movement of modules of genetic information within and between genomes. This has been observed in Burkitt Lymphoma cells, as an effect secondary to EBV infection. It is unlikely such a mechanism is regularly and commonly in use in human carcinogenesis.	Klein,1981; Newmark,1982;and Sager,1979.
Insertional Mutagenesis	Closely related to transposition, such a mechamism has been shown to be in action in human cancers. LINE-1 sequences insert into c-myc in a human breast carcimona; canine transmissible venereal tumour and a rat immunocytoma. Intercisternal-A particle genomes have been detected in c-mos gene causing the activaton of c-mos.	Morse et.al. 1988; Cohen et.al.,1983 and Rechavi et.al., 1988.

Gene Losses	Recessive genetic damage is significant because it releases negative regulatory controls. This can result in the activation of oncogenes. The inhibitory effects of the recessive genes or anti-oncogenes is lost too. Recessive mutations may also explain the hereditary nature of some cancers where no oncogenes are implicated, such as retinoblastoma and Wilms Tumour.	Cooper,1990; Green,1989; Ponder,1988; Knudson, 1971; Weinberg, 1990; Chen et.al., 1989; DeCaprio et.al.,1989; Van Heyningen et.al., 1985 and Porteous et.al., 1987.

E.Oncogenes.

An oncogene is a generic term which describes a gene which is capable of causing cancer/becoming a dominant transforming gene.

Abnormal or altered expression of such a gene directly causes the generation of the malignant phenotype. An oncogene i.e. a cellular oncogene is derived from a normal cellular gene called a proto-oncogene. There are also viral or v-oncogenes which are carried into the cell by an acutely transforming retrovirus and are transduced cellular oncogenes. It should be noted that the cellular origin of the oncogenes of DNA tumour viruses is as yet unknown.

The area of oncogenetics is somewhat outwith the scope of this thesis but it is reviewed in the following: Varmus,1984; Slamon et.al.,1987; Bishop,1991 (a) and (b); Cooper, 1990; Garrett,1986; Nishimura and Sekiya,1987 and Verma, 1986.

Chapter Three:
BREAST CANCER.

3.1. Introduction.

Hippocrates first wrote about the treatment of breast cancer in the fourth century B.C. Breast cancer however, remains a major killer of females-in fact the world wide age related data on the incidence of the disease shows that it is on the increase. In spite of the fact that this form of cancer has been studied over many centuries and that considerable sums of money and effort have been expended trying to elucidate it's molecular nature, the causes of breast cancer remain largely unknown. Moreover, there has been no substantial improvement in therapy and there is still no uniform approach to the treatment of the disease.

Some statistics reveal the extent of the problem: in the U.K., there will be some 26,000 diagnoses of breast cancer and approximately 16, 000 deaths per year from the disease. It is the commmonest cancer in females in the U.K. as a whole, with about nineteen percent of all new cases of cancer in females being cancers of the breast. In the U.S.A. and Europe it is the commonest cause of death of women in the 35-55 age group. A European or American female has a one in twelve chance of contracting the disease (Franks and Teich,1986 and Baum,1982).

3.2.Structure of the Breast.

The human breast develops in the sixth week of foetal life. Mammary ducts develop by growing down through the skin's layers (Baum, 1982). The mature breast is composed of varying proportions of secreting glandular tissue and adipose tissue.

The glandular tissue is divided into fifteen to twenty lobes (Townsend,1980). Each lobe contains hundreds of lobules which are connected by ductules which join together close to the nipple to produce the major lactiferous ducts which dilate into the lactiferous sinuses (Baum, 1982). They narrow again as they pass through the nipple to form the seven or so duct openings.

Breast tissue is enveloped in two layers of fibrous tissue: the deep layer overlying the muscles and the thin superficial layer beneath the skin (Townsend, 1980). Both of these layers of tissue are joined by thin fibrous ligaments called Cooper's Ligaments (Baum, 1982) which support the breast against the chest. Commonly tumours infiltrate and cause these ligaments to shrink so causing the overlying skin to pucker. This is clinically observed as peax d'orange.

The functional breast is a secondary sexual characteristic of the female. It functions principally as an

endocrine target organ and mediates lactation. Lactation is brought about by complex interactions of a number of hormones in the cells of the gland.

To do this the breast tissue is exquisitely sensitive to changes in it's hormonal environment, in a long term and a short term manner throughout the life of the individual female. Such changes can also be prompted by specialised conditions such as pregnancy, lactation and the consumption of exogenous hormones.

The breast can therefore be thought of as a collection of interacting cells which have a diversity and specialization of phenotypes.

3.3. Types of Hyperproliferative Breast Disease.

Conditions of breast hyperproliferation can be catergorised in the following way:

BENIGN BREAST DISEASES.

Type of Condition.	Example(s)of the Condition.
Inflammatory Disease	Puerperal Abscess, Periductal Mastitis, Tuberculosis.

Trauma		Haematomas, Fat Necrosis.
Developmental		Supernumary Breast(s); Absent Breast; Asymmetric Development.
Nipple		Paget's Disease.

MALIGNANT BREAST CONDITIONS.

Of Ductal Origin	Pre-Invasive	Intraductal Cancer.
	Invasive	Invasive Ductal Cancer Scirrhous Cancer.
Of Lobular Origin	Pre-Invasive	Lobular Carcinoma In Situ.
	Invasive	Lobular Invasive Carcinoma.
Of Connective Tissue Origin		Sarcoma.

(From Baum, 1982).

Cancers spread through local invasion and direct infiltration and metastasis. Spread occurs along the lymphatic channels and the blood stream. Both are connected so that a secondary tumour can become established anywhere in the body.

The most common site for secondaries to develop is in the regional lymph nodes from where the whole system becomes involved. Tumours in the inner quadrants of the breast can spread via the internal mammary chain of lymphatic tissue and into the

chest cavity (Baum, 1982). There, heart and lung can become involved and this leads to complications of fluid in the chest cavity or around the heart.

The most important mode of spread though is through the blood stream. This is probably due to direct invasion of cancerous cells into the veins draining the breast. Not all cells escaping are viable but those that are can lodge in the bone, liver, lung or brain. Secondary tumours in these locations can result in death from bone marrow failure, liver failure or respiratory failure (Franks and Teich,1986).

It is known that a malignant breast tumour is a number of different populations of cells with varying metastatic abilities. As has been previously pointed out (see section 2.1.), histological examination of such a tumour reveals that there is great morphological variation among cells of the same tumour for example in terms of karyotype, antigenicity, biochemistry, growth behaviour and susceptibility to chemotherapeutic drugs, radiation and heat (Baum, 1982).

3.4. Staging of Breast Tumours.

There are a number of tumour staging systems such as the Columbia, Manchester, American T.N.M. and International T.N.M. systems. The T.N.M. systems describe:

-Tumour size and attatchment to the underlying chest wall(T).

-Presence or absence of palpable lymph nodes(N).

-Presence or absence of distant metastases(M).

Using such schemes clinicians can assess patients in terms of their likely survival and the optimal regime of therapy to be adopted. Those with the best outcomes will have small tumours which are not attatched to the underlying substratum, with no lymph node involvement and no apparent metastases. This is called a stage one tumour.

The corollary of this is that a person who is catergorised as having stage four breast cancer has the worst outcome with a tumour which is quite large (greater then or equal to five centimeters); fixed to the chest wall, enlarged lymph nodes with secondary tumours detectable and ulceration of the breast itself.

Grading of tumours concerns the histological investigation of the tumour itself to determine the extent of cell de-differentiation. This is a very important factor in determining the course of theapy that the clinician will take.

3.5.1. The Molecular Biology of Breast Cancer-Introduction.

Epidemiological studies suggest that genetic factors are important in the development of breast cancer. It has been shown that if a close family member has or has ever been affected by breast cancer then an individual female is at risk of developing the disease too. A second linked factor is whether or not the disease in the relative occurred pre- or post-menopausally and if it was bilateral or not.

Case histories show that breast cancer often behaves a simple autosomal Mendelain trait. Finally, breast cancer is known to occur in families in association with other malignancies such as Ataxia Telangiectasias (Gatti et.al., 1991 and Morrell et.al., 1990). It is thought that some five percent of breast cancer cases are associated with an autosomal dominant allele (Van de Vijver and Nusse,1991 (b)).

There may also be significant genetical based predispositon to the development of breast cancer, as demonstrated by patients with Li-Fraumeni cancer syndrome. These indviduals in addiditon to having an increased overall cancer risk, may heve germ-line mutatons in the p53 gene which

predisposes to the development of breast tumours (Malkin et.al.,1990). A region of 17q21 has been implicated in the early onset of some forms of breast cancer too Hall et.al.,1990). This phenomenon will be discussed in section 3.6.2.-K..

Molecular biological techniques, especially the creation of transgenic mice models of breast cancer have helped the specific genetic alterations involved in the process of breast tumour development to be revealed. The most common lesion so far uncovered are:gene amplifications, gene deletions and mutated recessive oncogenes. Some rarer genetic lesions have also been discovered i.e. point mutations and gene translocations.

A. Amplification of Oncogenes-General Features.

Amplification of oncogenes (and other coding genes too of course)means that gene copy number is increased and as a result of this, the amount of the protein product is enhanced too (Chin et.al.,1991). A selective growth advantage might be conferred upon the cell depending on the exact nature of the gene product.

It is generally true that the actual gene is amplified along with an amount of surrounding DNA of approximately 100-1000K.B. This amplified DNA segment can often be visualised

and are called Homogeneously Staining Regions (where the amplified DNA is chromosomally loacted) or Double Minutes (where the amplified DNA is located extrachromosomally).

The amount of amplification of oncogenes varies between different studies and with certian clinoco-pathological perameters of the tumour. Considering only a few examples of amplification events:

Amplified Gene	Number of Cases Studied	Percentage Amplification	Reference
egf-r	21	14%	Ro et.al., 1989.
	189	2%	Slamon et.al.,1987.
	57	4%	Lacroix et.al.,1989
c-myc	176	4%	Tsuda et.al.,1985.
int-2/ bcl-1	46	9%	Zhou et.al.,1988.

B.Wnt/Int Gene Amplification.

This lesion is specifically associated with mouse mammary cancers-there is a poor association in the human

system (Van de Vijver and Nusse,1991(b)). WNT-1 (Human wnt-1 homologue)is not amplified translocated or even expressed in human breast cancers (Van De Vijver and Nusse,1991(b)).

C.Erb-B3 Amplification.

Erb B3 has been cloned via low stringency hybridisation from the human genome. It can be expressed at high levels in breast cancers-but whether it is amplified or rearranged is not at present known (Van de Vivjer and Nusse,1991(b)).

D.Neu/Erb B-2/HER-2 Amplifications.

Neu is homologous to but separate from egf-r (Schechter et.al.,1984 and 1985). The gene is amplified in approximately 9-30% of breast cancers and is also found to be amplified in adenocarcinomas of the salivary gland (Slamon et.al.,1987); stomach (Van de Vivjer et.al.,1987) and ovary (Zeillinger et.al.,1989).

Neu amplification correlates with increased levels of mRNA and protein and this is in turn associated with poor prognosis (Berger et.al.,1988) in most cases (King et.al.,1989(a) and (b)). This correlation is strongest in patients with lymph node metastasis (Ro et.al.,1989). This implies that increased levels of Neu are correlated with an increase in the growth rate but not metastatic potential (Van de Vivjer et.al.,1987).

In addition, in the case of non-invasive breast cancer neu overexpression is seen in about 70 % of cases of large cell comedo type ductal carcinomas in situ and 100% of cases of Paget's disease of the nipple (Van de Vivjer and Nusse,1991(b)). Neu amplification is, however never seen in small cell, papillary, cribiform ductal carcinomas in situ and benign breast cancer conditions (Van de Vivjer and Nusse,1991(b)).

In one study, however, neu was found to have little significant correlation with tumour stage (Lammie et.al.,1989). In another study as significant correlation between stage of the disease and HER-2/neu amplification was noted (Seshadri et.al.,1989).

A monoclonal antibody which has been raised against the extracellular domain of Neu can inhibit the growth of SKBR-3 cells which have an amplified egf-r (Drebin et.al.,1985). This

antibody does not affect breast cancer cells lines with a normal neu copy number, and so has been considered as a potential target for therapy in tumours which overexpress neu (Van de Vivjer and Nusse,1991(a)).

Donovan-Peluso's group have shown in addition that in metastatic breast cancer cells, amplification of myc, neu and int-2 is more marked than in non-metastatic cell (Donovan-Peluso et.al., 1991).

For example

	Non-Metastatic	Metastatic
myc	28%	43%
int-2	21%	40%
neu	24%	43%

(N.B.: these values refer to percentage of samples in which amplification of the gene in question could be detected).

The authors also found that gene copy number is associated with advanced disease, implying that these genetic alterations are in some way involved in the metastatic process.

E.Homogeneously Staining Regions.

One study suggests that these regions of chromosomes and also abnormally banded regions could be identified in 58% or so of the untreated primary breast tumours they analysed; most of these lesions localised to 8p1 (Saint-Ruf et.al.,1991). The authors conclude that such genetical alterations are formed in the 8p region and are moved elsewhere in the genome due to translocation event(s), so affecting tumour progression.

F.Chromosome 11 Amplifications.

Various studies have confirmed that amplifications of genes which reside on this chromosome are often observed in breast cancers. In the breast cancer cell line MDA-MB-134 (Cailleau et.al.,1974) the amplification of 2 genes here is described but it seems that neither is expressed which suggested that they may not be the genes which are crucial for this process (Lafage et.al.,1990).

G. Point Mutations.

Point mutations in the ras genes are rare in human breast cancers, but are common elsewhere such as in cancers of the colon, pancreas and lung (Van de Vivjers and Nusse,1991(a)).

H-Translocations.

A common karyotypic abnormality in primary human breast tumours involves the long arm of chromosome 1 (Van de Vivjer and Nusse,1991(b)).The actual role of this alteration is not known. Specific translocation events have not yet been reported.

Double minutes have been identified in approximately 40% of breast carcinomas and metastatic breast cancer cells in pleural effusions (Gebhart et.al.,1984). There is, however, not any evidence at present that these Double Minutes involve the amplication of any specific oncogenes. It should also be pointed out that there is a major problem in this type of investigation: having enough tumour material to be able to carry out the karyotypic analysis. This is the limiting factor in this area of research.

I.Deletions and Allele Losses.

Deletion of recessive oncogenes is now considered to be an important feature in the development of breast tumours. Loss of heterozygosity can be observed by searching for it at linked polymorphic DNA markers, that is to say by comparing breast cancer DNA and normal DNA form the same patient using Restriction Fragment Length Polymorphisms (RFLP's).

The main chromosomes that are affected are chromosomes 1; 3, 11, 13 and 17.

Chromosome 13 Allele Loss.

A study has found that there was loss of heterozygosity in 4 of the 14 cases studied (Lundberg et.al.,1983). Specific inactivation of rb in breast cancer tumours and cell lines has now been observed. In 2 of41 carcinomas (T'Ang et.al.,1988) and 15 of 77 carcinomas (Lee et.al., 1981) had rb alterations. In summary, rb inactivation is thought to be involved in about 20% of primary breast cancers (Van de Vivjer and Nusse,1991(b)).

Chromosome 17 Allele Loss.

Chromosome 17, at locus p13.3. shows the highest known level of allele loss in human primary breast cancer. 65% of informative cases have loss of heterozygosity here (Van de Vivjer and Nusse, 1991(b)).

The p53 gene lies in this region and it is a tumour suppressor gene. In 2 breast cancer cell lines, the retained p53 allele has no point mutations (Nigro et.al.,1989), which implies that the involvement of p53 and human breast cancer is far from being clearly understood.

Some breast tumours have a wild type p53 allele and a mutation acts in a dominant manner to inactivate the product of the wild type gene by forming a complex with it (Nigro et.al.,1989).

It should be noted however that some breast cancers have lost one p53 allele and have no mutations in the other one yet such changes are carcinogenic (Van de Vivjer and Nusse,1991(b)).

One group has found that 52% of the primary breast tumours they studied has loss of heterozygosity on the short arm of chromosome 17 (Smith et.al.,1983). Loss of heterozygosity here is associated with aggressive tumour behaviour and DNA aneupolidy and how p53 is involved in this process is not clear at present.

The alteration has been found to corrrelate with a high BrdUrd labelling index and DNA aneulpoidy (Chen etal.,1991), both of which are features of aggresive tumours.

Work by Chen's group suggests that breast cancers arise as a result of a hot-spot modification of chromosome 17, close to but not necessarily including p53 (Chen et.al.,1989). They concluded that other genes in this vicinity could be involved and that there may also be an other mechanism by which p53 becomes activated.

Mutated p53 polypeptide can also cooperate with ras to transform primary rodent fibroblasts (Jenkins et.al., 1985). Such data suggest that the half-life of p53 protein in being increased, in line with the above proposal.

Davidoff et.al. found that the presence of alleleic deletion(s) was not always associated with over expression of the protein (Davidoff et.al.,1991). Over expression of p53 was always associated with a mutation in breast cancers though.

If it is considered that an altered version of p53 could be involved in cell transformation in vitro even in the presence of wild type p53 (Jenkins et.al.,1985) then it implies that allelic loss at p53 could conceivably be needed or at least involved in breast cancer.

The theory proposed by Davidoff's group is that loss of heterozygosity involving p53 confers a growth advantage on the cell and this is selected for during subsequent tumour formation.

There is some evidence which seems to argue that p53 alterations alone are not sufficient to cause breast cancers to develop. Primary breast cancer needs perhaps more than one minimun deletion unit to be initiated (Coles et.al.,1990 and Sato et.al., 1990). There are mutations in p53 which are concurrent with 17p alterations, but which do not include the p53 locus (Coles et.al.,1990). So, it could be that such changes in the p53 genes are unrelated to p53 activation in breast cancer.

Chromosome 11 Allele Losses.

Losses in the 11p area of chromosome 11 are noted in approximately 20% of primary breast cancers (Theillet et.al.,1986). This region often involves c-Ha-ras alterations, but always involves the region between the beta globin and parathyroid hormone gene loci (Ali et.al.,1987). Such changes are associated with an oestrogen receptor negative status, large tumour size, grade three tumours and distant metastasis (Van de Vivjer and Nusse,1991(b)).

Chromosome 1 Allele Loss.

23% loss of homology has been demonstrated in primary breast tumours (Chen et.al.,1989).The actual loci involved are not known.

Chromosme 3 Allele Loss.

Some loss of heterozygosity is known here too(Van de Vivjer and Nusse, 1991(b)) and as for above, which loci lost are the critical ones is unclear.

J.Miscelleneous Genomic Alterations.

nm23.

nm23 is thought to be an anti-metastatic gene. Metastasis is a process which depends on the actions of a number

of genes (Fidler and Radinsky,1990). In this way nm23 was identified (Hennessey,1991).

The predicted amino acid structure of the gene product in humans is 78% homologous to awd of Drosphila. Mutation of this gene results in the generation of an abnormal tissue morphology, abnormal differentiation and necrosis (Rosengard et.al.,1989 and Dearolf et.al.,1988). In addition the gene resembles a nucleoside diphosphate kinase of D.discoideum (Wallet et.al.,1990). The protein product phosphorylates guanosine diphosphates and is involved in development, growth control and oncogenic tranformation.

If nm23 cDNA is transfected into murine tumours with high metastatic potential, the metastatic potential is reduced (Hennessy et.al.,1991). There are 2 human nm23 clones. One is lost with high frequency in breast cancers and hybridises to chromosome 7. One study has shown high levels of nm23 mRNA are associated with an absence of lymph node involvement in breast cancer (Hennessy et.al.,1991). The implication is therefore that nm23 reduced the ability of a tumour to metastasise (Bevilacqua et.al.,1989).

Epigenetic Effects.

AP1 proteins are factors which are vital for the transcription of certain genes (see Hames and Glover, 1988,

Bohman et.al., 1987 and Bos et.al., 1988 for reviews). A study analysing a number of primary breast carcinomas showed that all had enhanced levels of AP1 activity when compared to normal tissues (Linardopoulos et.al.,1990). It is thought that elevated levels of such transcription factors may be involved in carcinogenesis for example by increasing the rates of expression of certain important genes.

Chapter Four:
THE MOLECULAR BIOLOGY OF EGF-R.

4.1.Purification of Epidermal Growth Factor Receptor.

A.The A431 Cell Line.

This cell line is central to the study of many aspects of EGF-R structure and function. It was derived from a human vulval epidermoid carcinoma (Fabricant et.al., 1977). In addition to it being readily available and present in large quantities. The significance of A431 EGF-R is two fold: firstly, these cells express extremely high numbers of EGF-R upon their surfaces. This facilitates purification of the receptor. Secondly, the A431 cells are thought to be a good model in which to study EGF responses in vitro (at least with respect to short term responses)for example, mitogenesis (Sawyer and Cohen, 1985). In the long term though, the way in which A431 cells respond to EGF may not accurately reflect the situation in "normal"cells since EGF causes a reduction in the rate of growth of these cells whereas EGF stimulates growth of "normal cells" (Gill and Lazar, 1981).

B.EGF-R Isolation From A431 Cells.

Affinity chromatography,using immobilized EGF (Carpenter et.al., 1991), and monoclonal antibodies (Haley et.al.,1987) was used initially to purify the receptor (Cohen et.al.,1980). A near homogeneous preparation was attained.

Treating the isolated EGF-R with cyanogen bromide and trypsin (Downward et.al.,1984) created a number of peptides which were used to generate oligonucleotide probes (Haley et.al.,1987), to identify clones which encode EGF-R. The probes were subsequently used to screen a A431 and placental cDNA libraries cDNA and amino acid sequencing was then carried out (Downward et.al.,1984).

There are two very important findings with far reaching consequences to come out of this work, firstly EGF dependent tyrosine kinase activity and ligand binding ability were found to reside in the same molecule (Ullrich et.al.,1984 and Carpenter,1985). At the time of this discovery, such an interpretation was novel and regarded with some cynicism. Secondly, by comparing amino acid and DNA sequences, certain areas of the EGF-R gene and protein were found to share a high level of sequence identity with the oncogenic protein of the Avian Erythroblastosis virus:v-erb-B (Todderud and Carpenter, 1989).This was an early example of a growth factor receptor having been converted to an oncogene.

C.Genetic Engineering of EGF-R.

Analysis of any protein or gene requires that it should be available in plentiful supply. Genetic engineering has allowed this problem to be tackled as in the example of the expression of a functional EGF-R in insect cells (Greenfield et.al.,1989). Farrow et.al. have also tackled this problem by chemically synthesising the DNA which encodes EGF-R, related peptides and individual domains of the receptor and cloning them into an appropriately designed plasmid, then expressing them in E.coli cells (Farrow et.al.,1989).

The products are fully functional and so can be used to assay various EGF-R activities, as well as allowing the determination of physical characteristics for instance by X-ray crystallography. This approach has also been taken for the human c-Ha-ras oncoprotein (De Vos et.al.,1988 and Tong et.al.,1989)

It should be pointed out that the DNA sequence can be manipulated so as to create different forms of the EGF-R protein which are of great value in studying what the protein's role in the cell may be (see Section 5). In addition, the conditions can also be controlled so that large amounts of the protein can be made by the host cells.

4.2.The Structure of EGF-R.

4.2.1.Introduction.

In the late 1970's and early 1980's purified EGF-R became available as described in the preceeding section. This led in 1984 to the derivation of the amino acid sequence of tryptic digests of this protein, and the eventual cDNA cloning of the receptor (Downward et.al., 1984 and Ullrich et.al., 1984). Such work, along with classical biochemical analyses of EGF-R like protease digestion of the polypeptide, has revealed that EGF-R is a transmembrane glycoprotein, which has several distinct domains or regions which can be defined both functionally and physically.

4.22.The Extracellular or Ligand Binding Domain.

This domain is encoded by the N-terminal 261 amino acid residues of the EGF-R molecule (Ullrich et.al., 1984). Although knowledge is at present relativley sparse, the importance of this domain cannot be under emphasised. The interaction of growth factor and growth factor receptor is central to the regulation of cellular proliferation. The ligand binding domain is therefore of primary importance in this critical event.

If the rules governing growth factor-growth factor receptor interaction can be understood, then there may be the

potential for intervention in this process. This may have wide implications, for example in the control and management of diseases which involve either one of the proteins.

The EGF-R ligand binding domain, are very unusual in being able to bind 2 different ligands. EGF-R can bind TGF-Alpha and EGF, with high or low binding affinity. How this is brought about is not known, neither is the way that separate cellular signals can be generated subsequently, and how they result in different physiological consequences. Some of the various theories that have been considered are:

1-Different tissue distribution of ligand and/or receptor.

2-Varying accessibiltiy of ligand and/or receptor.

3-The binding of the ligand by the receptor induces different conformational changes in the receptor.

4-The involvement of other proteins and/or cellular structures in ligand binding and/or later steps.

As previously stated, relatively little is known about the structure of the ligand binding domain of EGF-R and how it actually carries out EGF binding. It is though that the domain is composed of two motifs: L and S. They are repeated in the order:L1:S1:L2:S2 (Hsuan et.al., 1989) and that they are situated such that they form four domains: I-IV.

L-Motifs.

Secondary structure predictions suggest each L-domain has four or five repeating motifs which are composed of alternating alpha-helix and beta pleated sheet structures. Each alpha-helix or beta sheet is separated by a very highly conserved glycine residue. Since glycine residues are characteristic of reverse turns, it would seem that the two L-domains form a beta-barrel motif which is involved in ligand binding.

The L-domains are supported by the two S-domains, with the putative alpha-helices containing most of the extracellular matrices twelve potential N-linked glycosylation sites in the form of:-Asn-X-Ser motifs or -Asn-X-Thr motifs.

S-Motifs.

These domains are approximately one hundred and sixty amino acids long and are very rich in cysteine residues, possibly in the form of disulphide bridges. Alignment of the amino acid sequences of the S-motifs shows that there are three internal repeats of three cysteine residues with approximately identical spacing patterns. This is also seen in insulin receptor, c-erb-B2/Neu, Drosphila EGF-R Homologue, IGF I Receptor, NGF-Receptor and the LDL Receptor.

Domains I, II, III and IV.

Lax and his group, on the basis of internal amio acid sequence homology, have divided the ligand binding domain of EGF-R into four domains: I, II, III and IV (Lax et.al.,1988 and 1989). They also assigned the following characters to each domain:

Domain	Character
I	N-Terminal Domain
II and IV	Cys-Rich Domain
III	Homologous to I, Flanked by Domains II and IV

In a series of experiments it was demonstrated that domain III is the main ligand binding determinant (Lax et.al., 1988 and 1989). The human EGF-R molecule was affinity labelled with 125I EGF and a cyanogen bromide cleaved fraction which contained the crosslinked EGF molecule was subsequently identified (Lax et.al., 1988). The role of this ligand binding domain was then analysed using chimaeric human-chicken interspecies EGF-R's (see Riedel et.al., 1987). This approach is possible because the binding affinity of the human receptor is about three hundred times greater than that of the chicken receptor in the same cellular

background (Lax et.al., 1988).

The experiment showed that the chimaeric EGF-R behaves like a human receptor for example, with respect to the EGF binding and biological resonsiveness, if and only if human domain III is present (Lax et.al.,1988). This same group also showed that two monoclonal antibodies which prevent EGF binding to it's receptor also bind to domain III (Lax et.al.,1988).

In their analysis of the ligand binding domain of EGF-R, Avivi's group aimed to discover specific residues which may be involved in EGF binding by site directed mutagenesis (Avivi et,al.,1991). Although still in progress, the research has so far shown that human and chicken domain III's have about seventy five percent amino acid sequence identity i.e. only thirty five or so residues are responsibile for the generation of the high affinity ligand binding characteristic of human EGF-Rs. Human domain III can be divided into two halves: IIIA and IIIB. Each half contributes equally to the binding energy. Finally, the binding site is composed of non-contiguous regions of domain III, exactly which residues are involved though, remains unknown.

The situation is not exactly as clear cut as such reports may make it seem though, since domain I may also be involved in defining ligand binding specificity too (Lax et.al.,1989). The current working hypothesis, therefore, is that domains I and III

form a cleft with the two cys-rich regions in close contact with each other and also the plasma membrane. The cleft is the site at which the ligand binds (Wu et al., 1989).

EGF-R, therefore is equivalent to allosteric enzymes, where the binding of the ligand causes the interactions between neighbouring subunits to be changed so resulting in an allosteric conformational change (Wu et.al., 1989).

In addition to this, it has been found that truncated receptors with no ligand binding domain (i.e. which resemble v-erb-B) can dimerize at low concentrations of Triton X100 (Kwatra et.al., 1992)which implies that this domain is not involved in this process.

4.2.3.The Transmembrane Domain

This domain is comprised of twenty three hydrophobic amino acids (622-644) (Hsuan et.al., 1989). They form a five looped alpha helix which spans the plasma membrane only once. The role of this domain in the generation and transmission of a signal over the plasma membrane is unclear, that is to say, how can the binding of EGF to the extracellular portion of the receptor result in the activation of the tyrosine kinase of the receptor located intracellularly?. Opinion is divided as to what the function of the transmembrane domain might be in this process. The two competing theories are:

1. The Intermolecular Activation Model. Here, ligand binding results in increased EGF-R association via the transmembrane domains i.e. dimerisation which causes cytoplasmic domains to be activated.

2-The Intramolecular Activation Model. In this case, binding of ligand causes the extracellular domains to become repositioned which, in turn, causes the transmembrane domain to be relocated. This causes a conformational change in the catalytic domain, which causes tyrosine kinase activity to be enhanced

Controversy is great in this area of EGF-R research mainly because the evidence so far collected is somewhat ambiguous and so cannot definitely confirm or refute either model. The crucial question: does the transmembrane domain have a passive (in line with the intermolecular activation model) or an active (in line with the intramolecular activation model) role in EGF-R signal transduction has not therefore been answered satisfactorily. Modern recombinant DNA techniques have been applied to the problem too, some of these experiments are described below:

1-The transmenbrane domain is tolerant of a number of amino acid changes, for example, removal or insertion of DNA or changing the nature of the residues. Such changes do not mimick EGF actions or affect ligand induced signal transduction, i.e. a

mutated transmembrane domain can still stimulate EGF-R tyrosine kinase activity and effect cellular responses when significantly altered (Kashles et,al., 1988)

2-Chimaeric EGF-R with the transmembrane domain swaps with the equivalent domains of HER2/neu/PDGF-Receptor/Insulin Receptor showed that the origin of the transmembrane domain has no effect upon the signal generation by the chimaeric EGF-R. (Lee et.al., 1989)

3-Point mutations mimicking those which constitutively activate c-erbB2/neu do not activate the signalling mechanism of EGF-R. This suggests a passive role of this domain in this process and is therefore evidence for an intermolecular activation model.

The activating mutation of c-erbB2/neu is in the transmenbrane domain:V664Glu (Carpenter et.al., 1991 and Bargmann et.al., 1986 (a) and (b)). This suggests that this alteration results in constitutive activation of tyrosine kinase activity (Bargmann et.al., 1986 (a) and (b)). So, a structural change can have a profound effect of the activity of the receptor. Three transmembrane domain alterations were made by Carpenter's group to investigate this further (Carpenter et.al.,1991). The mutations either shorten this domain at the C- or N-terminus or introduce point mutations equivalent to those for c-erbB2/neu or

proline substitutions into the receptor protein. None of the mutant receptors showed constitutive enzyme activation or altered ligand dependent signal transduction.

Recently, EGF-R mutants have been created whereby the basic amino acids at the cytoplasmic face of the receptor have been either deleted or deleted and replaced with neutral amino acid residues (Yamane et.al., 1992). Removal of these residues in rodent fibroblasts had the effect of greatly inhibiting EGF dependent tyrosine phosphorylation of EGF-R. When EGF was added to these cells, the mutant receptor showed no morphological alterations as did cells containing the wild type receptor (Yamane et.al., 1992). The implication is, therefore that the basic residue in this domain of the receptor are very important in the transduction of signals by EGF-R, possibly through the topology of the receptor in the plasma membrane

In cell lysates, altering the lipid composition of the plasma membrane can modify ligand affinity and basal kinetic activity (Downward et.al., 1984), thus suggesting an active role for the transmembrane domain in signal transduction and confirming an intramolecular activation model for this process.

The question of EGF-R's tyrosine kinase activity and its activation will be discussed in Chapter 5.

4.2.4.The Juxtamembrane Domain.

This region, extending from residue 645 to 657 encodes a very basic stop-transfer sequence which acts during EGF-R synthesis following insertion of the N-terminal end of the receptor into the lumen of the Endoplasmic Reticulum, to anchor the transmembrane domain intracellulary (Schlessinger et.al.,1978). As well as performimg this vital function, it seems that the juxtamembrane domain could also play a very important part in controlling EGF-R activity.

EGF-R activity can be regulated by a number of external stimuli in a process known as Transmodulation (see Section 5.10.).A major way in which stimuli might act to achieve this is the protein kinase C dependent phosphorylation of T654. This phenomenon is though to provide a negative control mechanism for EGF induced mitogenesis, and will be discussed later (see Section 5.8.).

4.2.5.The Cytoplasmic Domain.

This domain extends from residue 690 to 940 (Hsuan et.al.,1989) in the carboxyl end of the EGF-R molecule, and can be thought of as consisting of two main parts: a kinase region and a C-Terminal tail region.

The kinase region catalyzes the transfer of terminal

phosphate residues onto tyrosine residues in various target proteins. EGF-R is therefore a polypeptide with intrinsic tyrosine kinase activty-it was in fact among the first hormone receptor to be shown to have such an ability. The catalytic domain determines:

1-Presence at the plasma membrane.(*)

2-Ligand binding characteristics(**).

3-Biologcal signalling(*).

4-Early and delayed responses(**).

5-Mitogenesis(*).

6-Cellular transformation(*)

7-The fate of internalised receptors(*)

[(*)=Prywes et.al., 1986; (**); Schlessinger et.al. , 1978 and Li et.al., 1991]

These diverse functions are absolutely dependent upon tyrosine kinase activity (see Chapter 5).

Certain motifs within this domain are highly conserved among the tyrosine kinase family, the prototype of which is the src gene product. They are those motifs which are involved in ATP binding and the phosphotransferase reaction:

- -GXGXXGX at 695-700 (Carpenter, 1987)
- -k at 721 (Carpenter,1987)
- -DGF at 831-833 (Carpenter,1987)
- -ALE at 858-860 (Carpenter,1987)

The kinase domain can therefore be though of as consisting of a number of different regions, each with their own specific function in EGF-R kinase activity. For example, lsy 721.

Affinity labelling and modelling of the ATP binding site of this domain (Russo et.al., 1985) showed that lys721 could form a salt bridge with an oxygen atom on the beta phosphate of ATP. In addition, the motif,-Gly-X-Gly-X-X-Gly- is twenty five residues N to K721 at 695-700, and is known to be highly conserved in nucleotide binding proteins. The theory is that this motif is involved in correct orientation of the ribose phosphate groups and the conserved G at 695 is involved in recognition of C2 and N1 atoms on the adenine moiety of ATP.

The vital role of K721 in EGF-R's tyrosine kinase activity is demonstrated by consideration of the oncogene of Fujiami Sarcoma Virus: v-fps (Weinmaster et al.,1986) and the site directed mutagenesis of K950. The later results in a protein that although is metabolically stable, has no kinase activity. This suggests that it was involved in the hydrolysis of ATP and the phosphoryl transfer reactions (Snyder et.al., 1985).

ATP binding might also involve the -Asp-Phe-Gly- motif at 831-833, with Asp forming an hydrogen bond with the N-group at position 6 of adenine and Phe side chain and adenosine's ring interacting via hydrophobic bonds.

The Hinge Region.

The hinge region has been postulated due to studies which showed that the EGF-R tyrosine kinase domain is sensitive to proteolysis by proteases such as trypsin, chymotrypsin, elastase and Caclium activated neural proteases (Calpain). These agents result in the production of two fragmnents:

i-20K.D.- contains the C-terminus plus the three autophosphorylation sites.

ii-150 K.D.-Contains the extracellular domain and the tyroine kinase domain.

This pattern of digestion has suggested that tyrosine kinase activity and the C-terminus are linked by a proteases sensitive flexibile hinge region (Carpenter,1987).

The C-Terminal Tail Region.

This is the part of the EGF-R molecule which demonstrates the greatest amount of amino acid sequence divergence (Yarden, 1990). As well as phosphorylating a number of different exogenous substrates, EGF-R tyrosine kinase can catalyse self phosphorylation too. This autophosphorylation occurs only at a number of specific tyrosine residues in this C-domain of

through the sequencing of phosphotyrosine containing tryptic digests of the EGF-R derived from A431 cells, which were labelled in the presence of EGF. There are 3 sites at residues number 1173 (P1), 1148 (P2) and 1068 (P3). Y1173 is the main site of EGF induced autophosphorylation of EGF-R in intact cells (not in vitro). The phenomenon of EGF-R autophosphorylaton will be discussed later (see Section 5.6.2.).

The C-termini of different protein tyrosine kinase type growth factor receptors have divergent amino acid sequences. C-terminal deletions could be involved in oncogenesis. Consider the example of v-erb-B.

The process of recombination of the sequence for the transmembrane and cytoplasmic domains of EGF-R into the Avian Erythroblastosis Virus genome results in the loss of certain C- and N- terminal residues, and an N-terminal trunaction (Downward et.al., 1984). The later is correlated with the induction of erythroblastosis by EGF. Newly isolated erb B transducing viruses (Gamet et al., 1986), however, can create different diseases, transform fibroblasts in culture and have different sequences at the C-domain i.e. the differences in the C-domain are correlated with the disease causing potential of v-erb B.

Brain tumours of glial origin often have elevated levels of EGF-R due to amplification and rearrangement of egf-r (Malden et al., 1988). In glioblastoma multiforme, selective amplification of

the Transmembrane Domain, Cytoplasmic Domain and various fractions of the EGF Binding Domain are known to occur, with the later being the most common. It is though that the selective amplification of the cytoplasmic domain results in the consitutive activation of tyrosine kinase activity which ultimately causes cellular transformation.

Finally, a recent study has shown that alterations in the Erb-B protein which are associated with oncogenic potential are those which affect the protein tyrosine kinase domain (Nair et.al.,1992).

4.3.Epidermal Growth Factor Receptor Homologues and Analogues.

A.The Secreted EGF-R.

This polypeptide is produced only by A431 cells (Carpenter, 1987). It is thought to result from the expression of the 2.8 K.B EGF-R mRNA which is created by the translocated and rearranged egf-r (Merlion et.al., 1990 and Hunts et.al., 1985).

The predicted amino acid sequence corresponds to most of the external binding domain of the EGF-R, but, approximately eighteen amino acids are of unknown origin at the C-terminal region. One contains a glycosylation site

(Hunter et.al.,1984). The transmembrane and cytoplasmic domains are entirely deleted and since this is the region which anchors the protein molecule in the plasma membrane, the molecule is secreted (Weber et.al., 1984).

What function this form of EGF-R might have is not known, but it binds EGF, both this and normal EGF-R are physiologically identical and the glycosylation patterns of both proteins are identical (Carpenter, 1987). This, therefore suggests that membrane anchoring of EGF-R within the Endoplasmic Reticulum and Golgi Apparatus do not seem to be vital for correct glycosylation and activation of the external ligand binding domainn of EGF-R (Carpenter ,1987). Further processing (as yet unidentified) of the protein may also take place.

B.Oncogenes.

A great deal of work (see Downward et.al., 1984 for a review) has demonstrated that EGF-R is related to a number of oncogenes such as c-erb-B2, HER 1, HER2 and neu. This relationship and its implications will be discussed in Chapter 6.

C.Drosophila EGF-Receptor Homologue Gene (DER).

DER was originally identified by analysis of the Drosophila melanogaster genome using the EGF-R tyrosine kinase domain as a cDNA probe (Livneh et,al., 1985). It was the first

tyrosine kinase receptor gene identified in Drosophila (Schejter and Shilo, 1989)and it resembles EGF-R and neu (Bargmann et.al., 1986 (a) and (b)).

The protein has 3 cys-rich domains in it's extracellular domain and there are 2 different forms due to alternative splicing at the N-terminus (Schejter and Shilo, 1989). Tyrosine kinase domains are, however, conserved (Price et.al.,1989 (b)).

DER is expressed throughout development, including at the early embryonic stage (Schejter and Shilo., 1989). The pattern of expression changes though as development proceedes:

1-Embryo: Uniformly expressed throughout the embryo.

2-Larva: Expression is restricted to dividing cells in the imaginal discs and brain cortex.

3-Adult: Expressed in the nervous system and brain and in females, ovarian follicle cells.

(Schechter et.al.,1979).

When DER gene is mutated to create homozygous der mutants, the embryos which are created have the following phenotype:deterioration of anterior structures, ventral dentrial bands are missing, germ band doesnot retract and the Central Nervous System mid-line pattern is absent. Complementation analysis showed DER muations are allelic to the flb mutation.

4.4.The EGF-R Gene.

4.4.1. Introduction

In humans, the EGF-R gene is located on the short arm of chromosome 7: at locus p14-p12, close to the p11 fragile site (Haley et.al., 1987). The gene spans some 100K.B. and is composed of 26 exons (Haley et.al., 1987 and Shimizu et.al., 1980, 1982). Intron 1 lies between exons 1 and 2 and contains the elements of the promoter region.

4.4.2.The EGF-R Gene Promoter.

The promoter of the EGF-R gene has no TATA or CAAT box (Haley et.al., 1987 and Ishii et.al., 1985 (a) and (b) and 1986)., but the promoter proper and its suurounding area are very rich inG/C residues (Johnson et.al., 1988 (a) and (b)). Such sequences are associated with an open chromatin formation, and represent areas of transcriptonal initiation (reviewed in Gross and Garrod, 1987; Marinus, 1987 and Dynan, 1989). In addition, the CCGCC sites are the binding motifs for the transcription factor SP1 (reviewed in Kadonaga et.al., 1987; Johnson and McKnight, 1989 and Briggs et.al., 1986). SP1 is involved in the selective transcription of genes (Dynan, 1989).

Such motifs and their presence in the egf-r promoter imply that it may be constitutively active (Kageyama et.al., 1988 (a)and (b)) i.e. it has some metabolic function. CpG rich promoters are known to be characteristic of constitutively active genes (reviewed in Tazi and Bird, 1990; Bird, 1987 and Frank et.al., 1991)with house-keeping functions. In such cases optimal transcription levels are achieved by transcripton factor binding causing the appropriate necessary protein-protein interactions (see Hames and Glover, 1988, Johnson and McKnight, 1989; Ptashne, 1986, Rich et.al., 1984 and Travers, 1989).

Another point to note is that in addition to these transcription initiating sequences, intron 1 may also be involved in the control of de novo transcription, and so subsequent translational events. This will be discussed in the following paragraphs.

CAT in vitro transcription assays (reviewed in Kingsman and Kingsman, 1986) have identified and allowed the eventual purification of a number of transcription factors which are believed to be involved in the control of transcription of egf-r. They are SP1, CTF, AP1, MLTF and TF II B (Kageyama et.al.,1988 (a) and (b)).

These nuclear proteins are essential for the optimal expression of egf-r. Kageyama et.al. have also reported that in A431 cells there is a novel transcription factor regulating the transcription of egf-r. It has been named ETF (EGF-R Specific Transcription Factor) (Kageyama et.al., 1988 (a)and (b)). The molecular weight is 27 K.D, and the fact that ETF binds weakly to DNA templates suggests that secondary elements may be involved in this process. Johnson et.al. showed by DNase1 foot-printing, gel retardation assay and exonuclease III protection assay that there are eight sites in the promoter which specifically bind nuclear factors : -457 - -440; -417 - -402; -305 - -286; -214 - -200;195 - 170;-139 - 126;110 - -84 and -39 - -23 (Johnson et.al., 1988 (a) and (b)).

The EFT binding site consensus sequence is CCCCCGGC (Imagawa et.al., 1987). It bears a strong resemblance to the consensus binding site for the transcription factor AP1:-YCSCCMNSSS- (reviewed in Bohman et.al., 1987; Bos et.al., 1988; Hames and Glover, 1988 and Ptashne, 1986).where: Y=T or C, S=C or G, M=A or C and N=Any nucleotide.

This site is known to be involved in gene expression induced by phorbol esters (Imagawa et.al., 1987), and the authors therefore conluded that ETF causes transcriptional acitvation via transcription factor binding (Imagawa et.al., 1987).

It must be noted, however that the molecular weights of the 2 proteins are very different:ETF being 120 K.D and AP2 50K.D. (Imagawa et.al., 1987). ETF has, in addition, little or no effect of SV40 promoters in in vitro assays while AP2 strongly enhances SV40 transcription in such assays (Imagawa et.al. 1987). Such findings make it unlikely that such a mechanism of initiation of gene transcription is in action in this case.

A sequence identical to the ETF binding site has been located on the promoter of the human insulin receptor gene, but in the opposite orientation to that in the egf-r promoter (Araki et.al., 1987). This provides some evidence that ETF may be involved in the regulation of expression of genes other that that for EGF-R, importantly, other growth factor receptor genes. A second type of ETF has been characterized by Merlino's group (Merlion et.al.,1990). This ETF binds to a region of the promoter between nucleotides -139 and -126 and has been named ETF2.

Hudson's group isolated the 5'-region of egf-r from a human placental genomic DNA library (Hudson et.al., 1989). A region 1081 b.p. 5' to the ATG translational start site was found to be the important region with respect to transcriptional initiation and it's control.

Deletion analysis around this area further refined the region to a domain of 134 b.p. stretching from -19 to -153, and which is adjacent to the translational start point. The region contains the elements for:EGF, TPA, (Bu2) cAMP, Retinoic Acid and DMSO induction. It also has strong promoter/enhancer elements which as previouly stated are probably involved in basal and inductive responses (Hudson et.al., 1989).

Some viral proteins, principally those of Adenoviruses and Papilloma Viruses have been found to be involved in regulating egf-r expression by acting upon the promoter. Human Papilloma Virus 16 (HPV-16) protein E5 when added in combination with EGF to HeLa cells, causes an increase in the rate of proliferation of the cells in a soft agar assay system (Pim et.al., 1992). It would therefore seem that in some as yet unidentified way, the E5 protein is amplifying the EGF-R mitogenic signal. Their research has led the authors to believe that the most likely explanation is that E5 sensitized the cells to

any reduction in EGF concentration.

Group 3 type Adeonviruses seem also to be involved in regulation of egf-r promoter activity. The product of the E3 gene effects internalization and degradation of EGF-R i.e. mediates EGF-R down-regulation (Carlin et.al., 1989). The 2 proteins encoded by the E3 gene have molecular weights of 13.7 K.D. and 11.3 K.D. (Hoffman et.al., 1990 and Tollefson et.al., 1990).

Studies have shown that the 2 proteins form disulphide bonds, and that these oligomeric strucutes become inserted into the plasma membrane of the infected cell in a non-virus dependent manner (Hoffman et.al., 1992). Through an at present unclear mechanism, EGF-R becomes down-regulated. Phosphorylation of the heavier polypeptide upon serine residues is known now (Krajcsi et.al., 1992) and it is possible that this is involved in this process in some way.

As well as factors which increase EGF-R expression, the gene experiences negative regulation. Two agents which are responsible for this effect are thyroid hormone and retinoic acid (Thompson et.al., 1992). The mechanism of action of retinoic acid is not well known at present, but thyroid hormone receptor binds to a 36 b.p. C/G rich area of the egf-r promoter and can also act as a minimal enhancer.

Davidson showed that the number of EGF binding sites and the amount of immunoprecipitable EGF-R protein and mRNA are directly related (Davidson et.al., 1987). This therefore implies that the control of egf-r expression takes place at the transcriptional/post-transcriptional level. Experiments to dertermine if expression is controlled at the transcriptional level when carried out for egf-r expression, showed that if EGF-R levels increase, a concomitant increase in the transcription rate is seen (Davidson et.al., 1987).

Two enhancer elements have been demonstrated in the egf-r (Meakawa et.al.,1989). 1 is located at residues -1429 to -1109 and the other down-stream in intron 1 about 200 b.p. down-stream of the transcription initiation start site, which is approximately 530 b.p. long (Meakawa et.al., 1989). Both enhancers act cooperatively. Enhancer binding proteins are known which suggests that these elements may also mediate cell-type specific expresion of egf-r (Meakawa et.al., 1989).

The down-stream enhancer can only act properly in the presence of the upstream enhancer (Meakawa et.al., 1989), and the upstream enhancer has twice as great an effect on egf-r promoter activity when placed down-stream of the promoter compared to activity associated with normal

positioning (Meakawa et.al., 1989). This is an unusual feature of the working of this enhancer since generally, enhancers are position and orientation independent (Reviewed in Hames and Glover, 1988 and Kingsman and Kingsman, 1988).

The down-stream enhancer elememt has 10 nuclear factor binding sites so far identified, by protection assays (Kingsman and Kingsman, 1988). The identity of these factors is not known though.

In terms of egf-r cell-type specific expression the preliminary evidence from Masakawa's group indicates that the 2 enhancers are involved in this process. For example, in MCF-7 cells, the down-stream enhancer causes repression of the gene's promoting activity while the upsteam enhancer has the same levels of enhancement on HeLa cells (Xu et.al., 1984).

4.4.3.Transcription of the EGF-R Gene.

Both normal and transformed cells express EGF-R mRNA of 2 types: a 10 K.B. and a 5.6 K.B. species (Hsuan et.al., 1989; Ullrich et.al., 1984 and Hunter et.al.,1984). The amount of each type of mRNA varies depending on the cell type (Hunter et.al.,1984). A431 cells produce, in additon to those above, a 2.8 K.B. species that encodes a secreted form of the receptor

(Hunter,1984). Both types of mRNA hybridise with egf-r cDNA, but how they may be related is not known.

Transcription is initiated from multiple sites within an 800 b.p. GpG island in Exon1 (Haley et.al., 1987), in a sense and an anti-sense direction. The anti-sense transcripts extend through the coding exon1 and are reduced in number in the 5' flanking region. It is possible that some anti-sense transcripts could hybridise with coding sequences in exon1 of mature egf-r mRNA and so reduce the level of it's translation. Such an approach to translational regulation is well known (see Simons and Kleckner, 1989 and Weintraub et.al., 1985), and so is a viable theory for the regulation of expression of egf-r.

The regulation of egf-r transcriptiom by protein kinase C will be discussed later, but it should be stated here that the effects of protein kinase C upon the initiation of egf-r are detectable within 60 minutes and at down-stream exons within 120-240 minutes. Assuming a theoretical value for the rate of transcription to be 30 nucleotides per second, then intron1 (18K.B.) should be fully transcribed within approximately10 minutes. This calculation implies that there is likely to be yet further levels of control. A possible explanation is that since this intron region is close to protein binding sites, the rate of

RNA elongation may be influenced by the rate of formation of protein-DNA complexes.

4.4.4.Translation and Post-Translational Modification of EGF-R.

Translation of EGF-R mRNA in vivo is accompanied by co-translaional glycosylation within the lumen of the Endoplasmic Reticulum (Carlin et.al., 1989). Firstly the twenty four amino acid N-terminal leader sequence is removed from the 134 K.D. precursor, generating a 135 K.D. polypeptide (Velu et.al., 1989). This process is inhibited by Tunicamycin (Hsuan et.al., 1989) results in the generation of a 1260 K.D. precursor (Carpenter, 1987). This precursor has only high mannose type oligosaccharide side chains.

The process which takes about two hours, and can be inhibited by Monensin. Here, a ten-eleven residue complex of N-linked oilgosaccharide chains are added to the 135 K.D. core polypeptide. This creates the mature 170 K.D EGF-R (Velu et.al., 1989).

Scanning the amino acid sequence of the growth factor receptor gene shows that there are fifteen possible sites at which N-linked oligosaccharides can be added: twelve are located in the extracellular domain and three are located in the cytoplasmic domain.

Complex chains are found at glycosylation sites close to the N-terminus and high mannose type chains are located at glycosylation sites distal to the N-terminus (Carpenter, 1987).

The glycosylation events described above are necessary for EGF-R to be able to bind EGF (Carpenter,1985 and Sleiker et.al.,1986). This is demonstrated by using Tunicamycin to block oligosaccharide addition. This results in the production of an EGF-R which cannot bind EGF. However, merely adding carbohydrate moities is not enough to cause the acquisition of this activity. Various post-translational processing events are also needed before the EGF can bind to EGF-R (Carpenter,1987). This post-translational acquisition of ligand binding activity is also demonstrated in insulin receptor (Ronnett and Lane, 1981. and Reed et.al., 1981).

Exactly how this process occurs is not known but it occurs about 20-30 minutes after the start of translation, late in the Endoplasmic Reticulum (Carpenter, 1987). It is thought to require disulphide bond formation in the cysteine-rich ligand binding domain of the EGF-R (Velu et.al., 1989).

The acquisition of ligand binding ability requires proper core oligosaccharide addition, initial glucose trimming

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The acquisition of ligand binding ability requires proper core oligosaccharide addition, initial glucose trimming

and activation in the Endoplasmic Reticulum (Velu et.al., 1989). The post-translational modifications which are probably responsible for these events are phophorylation, fatty acylation and disulphide bond formation or rearrangement within the receptor molecule (Velu et.al., 1989).

Swainsonine is a fungal alkaloid that prevents one of the Golgi mannosidases involved in EGF-R processing form working. In its presence, an immature form of EGF-R is produced, which has a molecular weight of 160 K.D (Soderquist and Carpenter,1984). This species of EGF-R can be digested with endoglycosidase H to a 130 K.D aglyco form of the receptor.

The immature receptor seems to be formed normally but is not retained within the cell but is instead processed to the surface of the cell (Velu et.al., 1989). The EGF-R activities of 125I-EGF binding and stimulation of DNA synthesis are carried out as efficiently by this immature EGF-R as they are by the normal form of the receptor. The Swainsinine dependant form of EGF-R is, however, transported to the plasma membrane as described above. It can also bind EGF and has an active tyrosine kinase (Carpenter et.al.,1987).

Final processing of EGF-R occurs in the Golgi Apparatus. Here, mannosyl residues are removed and terminal

sugar residues such as fucose, sialic acid, galactose ans N-acetylglucosamine are added to the precursor core structure (Velu et.al., 1989).

In addition to glycosylation being vital in intracellular routing of EGF-R (Sleiker et.al., 1986), tyrosine kinase activity of the receptor seems also to be acquired post-translationally, but somewhat earlier than EGF binding ability is acquired (Hsuan et.al., 1989).

The initially synthesised 160 K.D precursor is therefore processed to the mature form of the receptor by capping of it's oligosaccharide chains with terminal sugars. Addition of core oligosaccharides seems to by vital in the correct functioning of EGF-R, that is to say, ligand binding activity, intracellular routing of EGF-R, tyrosine kinase activity and autophosphorylation of EGF-R.

The acquisition of autophosphorylation activity by the EGF-R protein (Slieker et.al., 1986) is particularly intriguing as the N-linked glycosylation sequence:-Asn 1020/1070/1124- is located in the molecule such that its position would prevent the glycosylation of the required residues (Kornfeld and Kornfeld, 1985). That glycosylation is involved in the acquisition of tyrosine kinase activity is demonstrated by the finding that

the 130 K.D receptor generated in the presenece of Tunicamuycin is not phosphorylated upon tyrosine residues in in vitro assays (Soderquist and Carpenter, 1984). Such evidence has been taken to support the intramolecular model for the activation of EGF-R tyrosine kinase activity

(Biswas et.al., 1985 and see section 5.3.2.).

4.4.5.Synthesis Associated Phenomena.

A-Receptor Down-Regulation.

Ligand binding causes receptor internalization (see Section 5. 11.6-B). Experiments with labelled EGF (Earp et.al., 1986 and 1991)have shown that down-regulation reduces greatly the cell surface binding capacity for EGF and usually leads to ligand degradation and possibly receptor degradation too (see Section 5.11.6-E).

In the WB cell line, Northern analysis shows that on addition of EGF,EGF-R mRNA levels increase by about 3-5 fold (Carpenter et.al., 1987). This suggests that in the WB line at least, the action of EGF that leads to EGF-R degardation is counter-balanced by a positive effect on receptor synthesis. This is also seen at the protein level. EGF-R synthsis can therefore be viewed as a regulated process. EGF-R levels are known to vary at different times during the cell cycle at

different cell densities and following retinoic acid induced differentiation.

The homeostatic regulation of EGF-R synthesis by ligand action may be a general phenomenon, for example, in human skin fibroblasts (Yarden and Ullrich, 1988). Other examples include IL2 which increases IL2 receptor synthesis in T-lymphocytes, prolactin and the prolactin receptor and insulin and insulin binding enhancement during preadipocytic differentiation (Carpenter, 1987).

This area of EGF-R metabolism is discussed in Sections 5.11.B-E.

B-Receptor Amplification.

EGF-R is detectable immunohistochemically in all cell types, except in those cells of the haemopoetic lineage. Overexpression of EGF-R is a significant feature and has been observed in many different types of cancers such as carcinomas of the breast, liver, bladder, and pancreas also in glioblastomas and sarcomas. Amplification of EGF-R is also seen in cell lines. The best example of this are A431 cells; an epidermoid carcinoma cell line which has approximately six million EGF-Rs upon its cell surface (See Section .1-A).

Different mechanisms increasing the numbers of EGF-R may be in use under different circumstances: amplification of egf-r; increase in the rate of transcription of egf-r and increase in the rate of post-transcriptional processing. The relative contribution of each to tumorigenesis is not known.

4.4.6.Regulation of Expression of EGF-R.

Regulation of the expression of EGF-R can be efected by a number of different agents, one of which is EGF. Adding EGF to a reposnsive cell leads to a rapid and dramatic reduction in the number of EGF-R at the cell surface, in a process known as receptor down-regulation (Carpenter,1987 and see section 5.11-B). In the non-transformend cell line WB (rat liver epithelial cell line) (Tsao et.al., 1984), among the EGF dependent effects are: increase in DNA synthesis, alteration of the activity of some glycolytic enzymes, increase in EGF-R tyrosine kinase activity, EGF-R internalization and degradation and an increase in EGF-R synthesis.

It has also been suggested that Protein kinase C has a role in the EGF dependent increase in EGF-R numbers (Bjorge and Kudlow, 1987). Protein kinase C is thought to be the receptor for the tumour promoting phorbol esters like TPA (Niedel et.al.,1983). TPA causes an increase in EGF-R mRNA in

KB cells (Clark et.al., 1985), and EGF-R synthesis in MBA 468 human breast cancer cells. This suggests that EGF requires protein kinase C involvement at some stage in it's action on EGF-Rs. How this may be brought about is unknown.

Regulation by Oestrogens is known too. Reports that 17-beta oestradiol causes an increase in EGF-R levels have been accumulating over many years. For example I 125-EGF binding studies in immature rat uterine membranes which have been treated with 17-beta oestradiol show a 3-fold increase in the numbers of EGF-Rs at the cell surface within approximately twelve hours. This is likely to be due to de novo synthesis, as Actinomycin D prevents it form happening (Mukku and Stancel, 1985 (a) and (b)).

Another example is in the case of mature rats, which have been ovariectomised, 17-beta oestradiol causes a 2-3 fold increase in EGF-R numbers in the uterus approximately eighteen hours after it's administration. When non-castrated animals were used at different stages of the oestrus cycle, the following observations were made:

Stage	Number of EGF-R
Metestrus	Low
Diestrus	Double the metestrus value
Proestrus	Maximal
Estrus	Low

These observed changes are equivalent to those noted for plasma oestrogens (Yoshida et.al., 1990)and for occupied nuclear oestrogen receptor levels (Clark et.al., 1989). This therefore suggests that there is an link between oestrogen receptors and EGF-Rs such that increased levels of oestrogens are correlated with increased number of EGF-Rs in the uterine membranes of mature and immauture rats (Gardner et.al., 1989).

The models which have been put forward to explain how oestrogens might affect EGF-R levels are as follows. Firstly, Oestrogen may cause general uterine growth which will as a natural consequence mean an increase in the numbers of EGF-R (Gardner et.al., 1989). Oestrogen on the other hand might prevent EGF-R down-regulation so that the numbers of EGF-R are always sufficient to stimulate growth. Finally, non-transcriptional mechanisms may cause the release of EGF

from EGF-R and so resulting in the generation of free EGF-Rs (Clark et.al., 1989; Earp et.al., 1986 and 1991).

Certain other points must be considered. The increase in receptor numbers occurs before tissue DNA synthesis begins (Kaye et.al., 1972 and Stancel et.al., 1990). Additionally, EGF causes myometrial contractions due to the release of arachidonic acid from sites within the uterine membrane (Stancel et.al., 1990). Uterine tissue has a large number of growth factor receptors in addition to those for EGF, for example insulin like growth factor I receptor (Murphy et.al., 1988). This receptor can be regulated by 17 -beta oestradiol.

In the normal uterus oestrogens are responsible for the regualtion of proto-oncogene expression, for instance, mRNA levels for the following proto-oncogenes are elevated by treatment with 17-beta oestradiol: c-myc (Travers and Knowler, 1987); c-myb (Murphy et.al., 1988); c-Ha-Ras (Travers and Knowler, 1987) and c-erbB (Downward et.al., 1984). This suggests that 17-beta oestradiol and a stimulatory effect upon, the growth of some cells, but this effect is not exclusively due to EGF-R.

That other agents may be involved in the oestrogen dependent growth of breast cancer cells was demonstrated in

the following way. MCF-7 cells (ER+)were treatred with the anti-oestrogen Tamoxifen (Cole et.al., 1986 and Levine et.al., 1985) to inhibit their growth and provide a synchronised population of cells. Monoclonal antibodies against EGF-R were found to block EGF/TGF-alpha binding, but base-line DNA synthesis and growth of the cells was unaffected (Arteaga et.al., 1988).

The conclusion that TGF-alpha/EGF is not the primary mediator of oestrogen mediated growth stimulatory effects was therefore reached. It is a likely possibility thought that complex interactions between a number of different factors may be involved or even other mechanism altogether such as different metabolic enzymes (Aitken and Lippman, 1985).

Additionally, in LNCap cells (a cell line deived from a lymph node carcinnoma of the human prostate gland) (MacDonald and Habib, 1992), EGF-R numbers are increased by 17-beta oestradiol in a dose dependent manner. This process is not inhibited bt Actinomycin D and Cycloheximide which implies that it takes place at the level of the protein, and not the egr-r gene (Schuurmans et.al., 1990). In this second example, it should also be noted that the same effect is achieved by treating cells with testosterone, progestrerone and triamcinolone acetonide too (Schuurmans et.al., 1990).

In a recent study of this phenomenon, cells were taken from normal, benign, and malignant human mammary tissue and cultured. Tritiated thymidine was used as a measure of the rate of cell growth (Gabelman and Emerman, 1992). Oestradiol was found to have no effect no cell growth, but EGF enhanced it's rate-this is in turn inhbited by introducing monoclonal antibodoes against EGF-R to the cells. Together EGF and Oestradiol were synergistic in 50% of cases (Gabelman and Emerman, 1992), which implies that the 2 signal transduction systems were interacting in some way at some stage.

Another likely model for hormonal control of EGF-R levels is that oestroegn enhances the stability of EGF-R mRNA. Increase in the rate of transcription of the gene ss possible too, so control may be mediated on a number of separate levels.

In male mouse livers, EGF-R levels are approximately 2-3 times lower than in female rat livers (Benveniste and Carson, 1985), and it has been proposed that androgens act directly to cause EGF-R induction as treating females with androgens leads to EGF-R levels incresing to male levels (Noguchi et.al., 1989).

Noguchi, by implanting testosterone into the spleens of test rats showed that the hormone caused a direct increase in the level of EGF-R mRNA and EGF binding ability in the liver (Noguchi et.al., 1989).

Exactly how testosterone maybe exerting it's effects on EGF-R expression is not known because the egf-r gene promoter does not have the androgen receptor responsive element consensus sequence -GGTACANNNTGTTCT- (Beato et.al., 1989). This does not rule out the possibility that testosterone causes induction of a substance which can interact with the egf-r promoter.

Phorbol esters cause positive regualtion of egf-r expression, even without protein synthesis. This may be due to RNA stabilization or de novo transcription. In A431 cells, serum deprivation leads to a decrease in the rate of egf-r transcription which is reversible upon the addition of phorbol esters or foetal calf seum i.e. agents which cause the induction of de novo transcription. The model currently accepted to explain this phenomenon is that phorbol esters activate protein kinase C. This results in the phosphorylation of EGF-R which reduces high affinity EGF binding, and intrinsic tyrosine kinase activty. The model is therefore in line with a positive feed-back system,

whereby EGF-R synthesis is stimulated by EGF-R down-regualtion.

EGF dependent increase in EGF-R synthesis is possibly due to a pathway equivalent to that of protein kinase C. In transient transfection assay systems, maximal stimulation with EGF and protein kinase C are not additive. This suggests that the 2 signal transduction pathways depend on certain as yet unidentified common factors. AP1 sites could be of significance here as AP1 is known to transduce signals generated by protein kinase C.

T3 is the most active form of Thyroid Hormone. T3 causes an increase in EGF-R expression at the mRNA level in the proximal tubules in the kidney (Humes et.al., 1992). In addition, it potentiatea the mitogenic response of these cells to EGF (Humes et.al., 1992), implying an integration point in the signalling pathways of these 2 receptor exists.

Dexamethasone causes a reduction in EGF-R synthesis at the mRNA level (Oberg and Carpenter, 1989) in foetal rat lung cells. This occurs without the need for protein synthesis, i.e. it is to do with the transcription of egf-r. Again, this shows that agents can regulate the expression of egf-r activity (Oikarineu et.al., 1989). The researchers concerned believed that an

increase in the rate of mRNA degradation is responsible (Oberg and Carpenter, 1991).

Dexamethasone could alternatively inhibit mitogenesis by blocking the G0-G1 phase (Ferradina et.al., 1992).

Differentiating agents may also affect the level of expression of EGF-R. Sodium butyrate, dimethylformamide and dimethylsulphoxide cause an increase in EGF-R mRNA and EGF-R levels in human colon carcinoma cells, and also in the SW620 cell line (Murphy et.al., 1986 and 1987).

Alpha interferon is one of the many proteins derived from leucocytes. Some of the effects are anti-proliferative, and are thought to involve down-regulation of EGF-Rs such as is the case for the insulin receptor (Pfeffer et.al., 1985 and 1987) and the transferrin receptor (Besancon et.al., 1985). This down-regulation of EGF-R is observed in human fibroblasts (Zoon et.al., 1986).

In the cell line CaKi-1, which is EGF dependent, there are high numbers of EGF-R at the cell surface. Alpha interferon blocks EGF dependent cellular proliferation and causes the reduction of receptor numbers at the membrane (Eisenkraft et.al., 1991). A reduction in receptor biosynthesis is thought to be responsible for these changes along with a partial block

of the elongation phase of translation demonstrated by polysome distribution on cellular EGF-R mRNA (Levine et.al., 1990).

Chapter Five: EGF-R SIGNAL TRANSDUCTION.

5.1.Introduction.

Signal transduction is the summation of all the processes involved in the translation of changes which occur outside the cell, into a programmed alteration of intracelluar activities. Cells perceive such environmental changes through the receptors located in their plasma membranes, receptors also have a central role in the generation and propagation of those signals. In addition, since the very first event in the pathways of signal transduction is the binding of a receptor by it's cognate ligand, receptor-ligand binding is a major point of control of intracellular signalling.

When a ligand, for example a growth factor such as EGF binds to it's receptor, a cascade of biochemical events is initiated. These fall into three main groups:

1-Ion Fluxes.

2-Inositol Lipid Metabolism.

3-Activation of Protein Kinases which causes protein phosphorylations.

The purpose of this chapter is to assess the current literature on EGF-R signal transduction. How each of above listed events relates to EGF-R will be discussed, with particular emphasis being given to EGF-R's role as a tyrosine kinase.

5.2.EGF-R/EGF Binding.

5.2.1.Introduction.

A number of different ligands can bind to EGF-R:EGF: (1:1 ligand receptor stoicheometry (Weber et.al., 1984),TGF-Alpha;(Carpenter, 1987), Amphiregulin (Shoyab et.al., 1979) and Pox Virus Growth Factors (especially Vaccinia Virus Growth Factor) (Buller et.al., 1988).

Overall homology between these ligands is, however, low: approximetly 22% (Velu et.al.,1989), yet binding affinities remain about the same for each ligand.How this might be brought about is as yet unknown, but some current models will be discussed below.

Binding to EGF to EGF-R has pleiotrophic physiological effects (Carpenter, 1987). One of these effects is the activation of the intrinsic tyrosine kinase of EGF-R (Schlessinger, 1986 and see also Section 5.6.). As a result, a number of cellular proteins are phosphorylated, including EGF-R itself (see Section 5.6.1.).

Such tyrosine phosphorylations are believed (in part at least)to be responsible for the complex set of events which occurs both upon the surface of the cells and within the cell subsequent to EGF binding:EGF/EGF-R complex oligomerization, down-regulation, internalization, degradation and intracellular routing.

Such events have a central position in the EGF-R signal transduction pathway and will be discussed separately (see Sections 5.11.6.-A to 5.11.6-F.).

In order to have its effects upon the cell, EGF must be present and be bound to EGF-R uninterrupted for approximately 6-10 hours (Shechter et.al., 1989 and King and Cuatrecasas, 1982). It is during this period of time that the EGF-R metabolic events listed above take place and the cells ultimately becomes committed to DNA synthesis.

5.2.2.Quantitating Binding.

Analysis of ligand binding is carried out by Scatchard analysis of data generated in ligand binding assays (Leake et.al., 1987). Such analysis derives the two perameters:

-Affinity Constant: Kd.

- For the high and low affinty receptors, Kd values range from 10-9 to 10-10M (Carpenter et.al., 1987).

-Receptor Concentration: B max.

Cells generally have 20,000 to 200,000 EGF-R's upon their surface (Carpenter,1987). There are notable exceptions to this rule though (see Section 4.1-A on the A431 cell line) with differing affinities for EGF or EGF or EGF/EGF-R.

Scatchard analysis of EGF-R reveals that there are two distinct receptor populations: one with a high affinity and one with a low affinity for EGF. The high affinty population comprises about ten percent of the total receptor population, with the difference in Kd values between the two populations being 10-20 fold (Carpenter, 1987).

5.2.3.Populations of EGF-R's.

In addition to that evidence provided by Scatchard analysis, much work has recently been carried out which demonstrates that with respect to affinity for EGF, there are at least 2 groups of EGF-Rs.

In KB cells, under physiological conditions, there are two types of EGF-R: high and low affinity receptors (King and Cuatercasas, 1982). Such work was made possible by the development of monoclonal antibodies (Bellot et.al., 1990) :mAB108 was raised against the extracellular domain of EGF-R in NIH 3T3 cells. It specifically reduces high affinity binding of EGF, Kd and number of receptors remains unchanged.

On treating cells with antibody at low concentration leads to a 50-100% reduction in early responses (Bellot et.al., 1990) and shows high affinity binding is dependent upon the on

rate of EGF binding. So, high affinity binding seems to be essential in signal transduction. (This was later confirmed by Defize et.al., 1989).

2E9 specifically blocks EGF binding to low affinity receptors. Adding it to intact cells has no effect on EGF-R tyrosine kinase activity and early responses (Defize et.al., 1989) implying that these are due to high affinity type EGF-R mediated events.

In HeLa cells, the situation seems to be more complicated with three different EGF-R sub-groups having been identified. Studies using the monoclonal abtibody mAb108 and 2E9 and PMA (which blocks low affinity receptor acitvity specifically) revealed that the low affinity receptor group are of two types and high affinity receptors of one type (Berkers et.al., 1991)

Class	Affinity	Association Constant	Dissociation Constant
I	High	6.2x105 M-1S-1	3.5x10-4 S-1
II	Low	3.3x106M-1S-1	8.0x10- S-1
III	Low	3.2x104 M-1 S-1	1.6x10- S-1

A monoclonal antibody: 13A9 has been created against EGF-R which almost totally prevents TGF-alpha binding to EGF-R but has little or no effect upon EGF binding to EGF-R. Using partially purified receptor, Winkler's group demonstrated that

with h13A9 (i.e. no TGF-alpha binding)EGF-R tyrosine kinase activity is increased about 2.5 fold compared to a 4 fold increase with EGF alone (Winkler et.al.,1989).

Although highly homologous to human EGF-R, chicken EGF-R binds mouse EGF with approximately 100 fold less affinity than the human receptor does. Human TGF-alpha binds with about the same affintiy to both chicken and human EGF-Rs but for chicken EGF-R, human TFG-alpha is about one hundred times better at stimulating DNA synthesis than human EGF (Lax et.al., 1988).

This type of evidence, may suggest that as well as there being a number of different kinetically identifiable EGF-R sub-populations, binding of EGF by different ligands has separate effects upon intracelluar activities. The models currently put forward to explain the discrepancies are as follows:

1-TGF-alpha and EGF bind to different parts of the EGF-R molecule. Binding of one or the other of the ligands may result in and/or cause, displacement of the other ligand or change in receptor configuration.

2-TGF-alpha and EGF when they bind to EGF-R do so at the same site, but cause different conformatinal changes in EGF.Binding is therefore competitive.

3-The different effects upon the second messenger molecule Ins 1,4,5,trisphosphate and tyrosine phosphorylations (Korc et.al.,

different cell densities and following retinoic acid induced differentiation.

The homeostatic regulation of EGF-R synthesis by ligand action may be a general phenomenon, for example, in human skin fibroblasts (Yarden and Ullrich, 1988). Other examples include IL2 which increases IL2 receptor synthesis in T-lymphocytes, prolactin and the prolactin receptor and insulin and insulin binding enhancement during preadipocytic differentiation (Carpenter, 1987).

This area of EGF-R metabolism is discussed in Sections 5.11.B-E.

B-Receptor Amplification.

EGF-R is detectable immunohistochemically in all cell types, except in those cells of the haemopoetic lineage. Overexpression of EGF-R is a significant feature and has been observed in many different types of cancers such as carcinomas of the breast, liver, bladder, and pancreas also in glioblastomas and sarcomas. Amplification of EGF-R is also seen in cell lines. The best example of this are A431 cells; an epidermoid carcinoma cell line which has approximately six million EGF-Rs upon its cell surface (See Section .1-A).

a-EGF binding to low affinity receptors causes de novo synthesis of high affinty type receptors.

or,

b-The two populations have different turn-over rates, with that for high affinity receptors being faster.

or,

c-Expression and/or activity of high affinity receptors requires the presences of an effector molecule which turns-over rapidly snd is in some way involved in the generation of different EGF-R populations.

5.2.4.The Regulation of EGF-R/EGF Binding.

As previously stated, ligand-receptor interaction is a crucial control point in the process of signal transduction. A number of agents are currently believed to regulate this interaction.

Table Two: Agents Which Regulate EGF/EGF-R Binding.

Agent	Reference
n-Butyrate	Gladhaug et.al., 1992.
Phorbol Esters	Shoyab et.al., 1979.
Photoactivated Psoralens	Geordias and Laskin, 1991.
DiMethylSulphOxide	Vessey et.al., 1991 and Baker et.al.,1978.
Alkyl-Lysophospholipids	Kosano and Takatan, 1988.
Vitamin K (and other quinine derivatives)	Shoyab et.al., 1979.
Teleocidin B	Imani et.al., 1980.
Saccharin	Lee et.al., 1981.
Cyclamate	Lee et.al., 1981.
Prostaglandin f2-alpha	Jimenez de Asua and Goin, 1992.

(N.B. Prostaglandin f2-alpha affects phosphorylation status too see Felder et.al., 1992).

A.Phorbol Esters.

Phorbol esters, especially T.P.A., and related plant macrocyclic diterpenes inhibit the binding of EGF to EGF-R, for example in mouse skin (Lee and Weinstein, 1978 9 (a) and (b)), HeLa Cells (Lee and Weinsein, 1978 (a) and (b)) and R6PKC3 Rat Fibroblasts (Geordiadis and Laskin, 1991).

The potency of the amount of inhibition is correlated with the effectiveness of the agent to promote tumours (Lee et.al., 1981). It is believed that binding to high affinity type receptors is blocked specifically by phorbol esters (King and Cuatrecasas, 1982).

The current model explaining the effects of tumour promoters upon EGF/EGF-R binding is as follows. TPA binds to it's intracellular receptor: Protein kinase C (Nishizuka, 1984). Protein kinase C is as a consequence activated and phosphorylated in addition to other proteins, EGF-R (Jetten et.al.,1985 and Cochet et.al., 1984 (a) and (b)). This phosphorylayion of EGF-R in some way, causes a conformational change within the ligand binding domain of EGF-R so that EGF can no longer bind/does so with less affinity (Lee et.al., 1981 and Shoyab et.al., 1979).

B.The Involvement of PDGF.

PDGF can enhance EGF's mitogenic effect in vitro (Leof et.al., 1982 and Olashaw et.al., 1982) by possibly reducing high affinity binding (Wrann et.al., 1980 and Heldin et.al., 1982 and Wharton et.al., 1982), thus converting high to low affinity EGF-R's (Bowen-Pope et.al., 1983 and Collins et.al., 1983).

PDGF also stimulated protein kinase C activity. In protein kinase C down-regulated cells (Olashaw et.al., 1986), PDGF can still effect a reduction in EGF binding (Davis and Czech, 1987). Studies with human foreskin fibroblasts show in the presence of PDGF, T654 is not phosphorylated and PDGF results in a reduced level of EGR high affinity receptor binding, which suggests that PDGF dependent lowered EGF binding depends upon protein kinase C (Olsen and Pledger, 1990).

C.The Involvement of sn-1,2-Diacylglycerol.

sn-1,2-Diacylglycerols, reduce EGF binding to EGF-R and induce expression of Ornithine Decarboxylase gene (OCDase), an event which is correleted with tumour promotion (Verma et.al., 1979).

These effects occur in a dose and time dependent manner, and in parallel with the effects of phorbol esters (Jetten et.al., 1985). The diacylglycerols, which activate protein kinase C the most, also have the greatest effect in terms of reducing EGF binding, and OCDase induction, and also can associate with the plasma membrane and enter the cell with ease.

D.Non TPA-Tumour Promoters.

In terms of modulation of EGF /EGF-R binding, the most significant of the group of non-TPA-phorbol esters is Palytoxin.

In Swiss 3T3 cells, this agent has been demonstrated to reduce EGF binding to both low and high afinity EGF-R's in a protein kinase C independent way (Olsen and Pledger, 1990 and Wattenberg et.al., 1989) although the mechanism of reduction is not clear. It is thought to mediate this effect by inducing a sodium influx into the cell (Wattenberg et.al., 1989)which reduces EGF binding (Wattenberg et.al., 1989).

In Swiss 3T3 and Balb/c/3T3 cells, however,stimulation with PDGF or TPA reduces EGF binding in a manner which is not dependent upon external sodium concentrations. It is therefore likely that TPA and non-TPA phorbol esters have different mechanisms for reducing EGF binding to EGF-R.

E-Photoactivated Psoralens.

Psoralens activated by ultraviolet-A light (Laskin and Lea, 1991) reduce binding of EGF to EGF-R (Mermelstein et.al., 1989 and Laskin and Lea, 1991). Binding is, however, reduced to a lesser extent than that induced by phorbol ester tumour promoters. How the reduction is brought about is unclear, but it is thought that phosphorylation of EGF-R by photoactivated psoralens is involved. In addition, these agents seen only to bind to high affinity type EGF-R's (Yurkow and Laskin, 1987).

Geordias and Laskin have found that cells of the rat fibroblast cell line R6C1 (control cells) when compared to the same cells transfected with a retrovirus enncoding the beta-1 form of protein kinase C: R6PKC3 cells, the following can be observed (Geordias and Laskin, 1991): R6pkc3 cells were more sensitive to TPA than R6C1 cells, R6PKC3 cells do not recover from TPA dependent 125 EGF binding and finally, the twoclones were equally sensitive to phtoactivated psoralens.

This suggested to the above authors that TPA and photoactivated psoralens reduce EGF/EGF-R binding by different mechanisms, and that the photoactvated psoralen method does not involve protein kinase C. Features which complicate the interpretation of the experimental results are that it is to be

expected that different clonal isolates will have slighlty or even totally differing properties. Secondly, the R6PKC3, protein kinase C beta-1 containing cells have fewer EGF-Rs to start with.

F-Dimethylsuphoxide (D.M.S.O.).

In the human hepatoblastoma derived cell line:HepG2, DMSO cause a large reduction in the rate of growth, altered cellular morphology (cells are flat and spread), a reduction in the response to EGF at doses which do not effect EGF-R affinity (McGowan, 1988) and a large increase in EGF binding, without the corresponding inverse in proliferative reponse to EGF (Vesey et.al., 1991).

Again high affinty type EGF-R's are prevented from binding EGF (Gladhaug et.al., 1988).

G.Dexamethasone.

Dexamethasone is a synthetic glutocorticoid (Oberg and Carpenter, 1989). In diploid human foreskin cells, this compond alone has no effect upon their rate of proliferation but significantly enhances the mitogenic effect of EGF. This is brought about by an increase in EGF binding to EGF-Rs (Baker et.al., 1978). Binding analysis revealed that the number of receptors

stayed approximatley the same with and without dexamethasone treatment, suggesting that receptor affinity is increased by such treatment (Baker et.al., 1978).

5.3. EGF-R Transmembrane Signalling:The Activation of the Intrinsic Tyrosine Kinase of EGF-R.

5.3.1.Introduction.

Molecular biological analysis of EGF-R revealled that ligand binding and tyrosine kinase activities both reside in the same individual polypeptide molecule, and that the membrane spanninng domain of EGF-R crosses the plasma membrane only once (Carpenter, 1987). It is therefore important to explain how an extracellular signal can be transmitted over the plasma membrane so that the tyrosine kinase domain is activated.

There have been two main theories propounded over recent years to explain this process-their accuracy is the subject of much controversy, not least of all because an understanding of this process will be a tremendous advance in the wider field of cellular signalling.

Both models propose that ligand binding causes a change in the conformation of the extracellular domain of the receptor. The two current models are:

1.The Intracellular Activation Model.

This model states that EGF binding to the receptor causess a conformational change in the EGF-R molecule:a vertical dislocation of the transmembrane domain (Schlessinger, 1988). This causes that rate of catalysis of the intrinsic tyrosine kinase to be enhanced (Schlessinger et.al. 1978; Carpenter,1985 and Bertics et.al.,1988).

2.The Intermolecular Activation Model.

This model is based on the theory that EGF causes individual EGF-Rs to cluster or oligomerise. Interactions between neighbouring EGF-R tyrosine kinase domains stabilized by EGF binding leads to the activation of the kinase domain. No conformational changes are needed. This model is also known as the allosteric activation model or the allosteric oligomerization model (Yarden and Schlessinger, 1987 ; Schlesssinger et.al., 1978 and Carpenter,1987).

5.3.2.The Intramolecular Activation Model.

Evidence that the tyrosine kinase of EGF-R could be activated as a consequence of a conformational change(s) which occurs within the individual EGF-R molecule was first put forward by the Biswas group in 1985 (Biswas et.al.,1985). These investigators used solubilized and purified EGF-Rs from A431 cells. Their salient findings were firstly that glucose-gradient sedimentation analysis of the non-denatured solubilised receptors showed 2 EGF-R populations:

i:7.7 S - Monomeric.

- Active tryosine kinase.

-Tyrosine kinase acivity is not enhanced by EGF.

ii:12 S -Dimeric.

-No tyrosine kinase activity.

-Adding EGF results in the creation of monomers which have an active tyrosine kinase moiety.

Secondly, in vitro, the rate of EGF-R autophosphorylation does not depend on EGF concentration-that is to say that it follows zero-order kinetics. Finally, DTT treatment of the 12S form of the receptor showed that there were no disulphide bonds between the two receptors which form the dimer.

They concluded that:

1-Only monomeric receptors have an active tyrosine kinase.

2-Receptor-receptor interactions cause a decrease in kinase activity.

3-Monomers exhibit autophosphorylation i.e. it is an intramolecular process.

4-Monomers and dimers exist in a state of equilibrium. Addition of EGF increases EGF-R tyrosine kinase activity by shifting the equilibrium, towards monomers.

Sucrose-density gradient ultracentrifugation of purified EGF-R in low concentrations of salt and 0.25M ammonium sulphate showed that upon addition of EGF monomeric EGF-R are the most common species (Koland and Cerione, 1988). If this monomeric form of the receptor is isolated from the gradient and the tyrosine kinase activity assayed in the presence of 0.25M ammonium sulphate kinase activity is found to be dependent upon EGF concentration. How this may be effected is unknown (Koland and Cerione, 1988). In addition, non-denatured gel electrophoresis revealled that most autophosphoryated EGF-R is monomeric (Koland and Cerione, 1988).

Further evidence to support this model is that Sphingosine is known to be able to stimulate tyrosine kinase

activty of EGF-R (Northwood and Davis, 1988), in a manner which is not dependent upon EGF-R oligomerisation (Northwood and Davis, 1988).

Yarden and Schlessinger also report that autophosphorylation of EGF-R purified from A431 cells is parabollic with time compared to EGF dependent phosphorylation which is concentration dependent. This led to their proposal that autophosphorylation is activated intracellularly while EGF dependent EGF-R phosphorylation is activated as a result of an intercellular process. An important point to bear in mind is that autophosphorylation of EGF-R takes place upon various residues located in the C-terminal domain of the receptor (Carpenter, 1987 and see Sections 5.6. and 5.8.). This therefore focuses attention on how the tyrosine kinase and cytoplasmic domains might interact.

There are some significant pieces of evidence against the intramolecular tyrosine kinase activation model. This model requires that the binding of EGF to the extracellular doman results in a conformational change in that region and that the change is transmitted via a vertical dislocation of the hydrophobic transmembrane domain. This is, however,unlikely or maybe even impossible, when the following two points are considered:

a-The transmembrane domain is bordered by charged amino acids (especially important in this case are the prolines at the cytoplasmic face (Ullrich et.al, 1984)) on both faces of the plasma membrane, which will prevent on thermodynamic grounds its vertical movement within the plasma membrane (Schlessinger, 1988).

b-This region at the DNA and amino acid levels is very variable between species (Livneh et.al.,1985). It can also tolerate a variety of alterations and still remain able to effect signal transduction:

-Site directed mutagenesis to reduce the length and composition of the transmembrane domain (Gill et.al., 1985 and Bargmann et.al., 1986 (a) and (b))

-Generation of insulin receptor extracellular domain/EGF-R transmembrane domain chimaeras (Riedel et.al., 1986).

This suggests that the transmembrane domain may be essential but is not by itself sufficient for transmitting signals across the plasma membrane.

5.3.3.The Intermolecular Activation Model.

EGF binds to EGF-R and as a consequence, there is a conformational change in the extracellular domain. Purifed EGF-R extracellular domains have recently been found to undergo such conformational changes under these circumstances (Spaargaren et.al., 1990 and 1991). It is held by some that oligimerization is an inherent property of the ligand binding domain of EGF-R (Lax et.al., 1991). The ligand binding domain has two functions: EGF binding and oligomerization.

As a result of these changes in structure of the ligand binding domain affinity for EGF-R's is increased (Ullrich and Schlessinger., 1990). Cytoplasmic domains of adjacent receptors therefor interact and as a result, tryosine kinase activity is enhanced (Basu et.al., 1981).

Central to this model is the concept that EGF-R molecules have an intrinsic ability to form oligomers (Yarden and Schlessinger, 1987 and Honneger et.al.,1989). The theory is that oligomerization brings individual receptors into close proximity, and this therefore allows the tyrosine kinase domain of one receptor to phosphorylate appropriately the other receptor molecule.

Evidence for this model, at least in vitro, is provided by Honegger's group who co-expressed kinase positive and kinase negative EGF-R mutants in NIH 3T3 cells, which are cells that lack endogenous EGF-R's. Phosphopeptide mapping showed that the kinase negative mutant's autophosphorylation sites are phosphorylated and it is presumed that this is due to phosphorylation by the EGF-R containing the active kinase domain; that is to say cross-phosphorylation between the two types of receptor molecule (Honegger et.al.,1988, 1989 and 1990).

Although it is still formally possible that autophosphorylation is mediated either totally or to some degree by an intramolecular mechanism the above noted findings strongly support the idea that tyrosine kinase activity, and also autophosphorylation of EGF-R (Schlessinger, 1988), and as a result this casts doubt upon the validity of the intramolecular activation model.

Further Evidence for the Intermolecular Activation Model.

1-Without EGF, receptors exist as monomers (Northwood and Davis,1988).

2-EGF-R dimers have greater kinase activity and EGF affinity than monomers (Yarden et.al. 1985 and Boni-Schnetzler and Pilch, 1987).

3-EGF induces EGF-R clustering:

-On the plasma membranes of intact cells

-Demonstrated biophysically (Zidovetzki et.al., 1981 and Hillman and Schlessinger, 1982).

-Demonstrated morphologically (Haigler et.al., 1978)

-Demonstrated biochemically (Fanger et.al., 1989 and Bonni-Schnetzler and Pilch, 1987).

-In plasma membrane preparations (Spaargeren et.al., 1990).

-In pure EGF-R preparations (Yarden and Schlessinger, 1987 and Lax et.al., 1989).

4-Monoclonal antibodies against EGF-R activate the kinase domain of the receptor and stimulate DNA synthesis in quiescent cells (Carpenter, 1989).

5-Cross-linking with antibodies or lectins or chemicals results in enhancement of phosphorylation and also mitogenesis (Schreiber et.al., 1983).

6-Immobilization of EGF-R prevents EGF activation of kinase activity (Yarden and Schlessinger, 1987).

7-Induction of EGF-R dimerization by anti-EGF-R antibodies is correlated with EGF-R kinase activation (Spaargaren et.al., 1990), and visa versa.

8-neu, which is closely related to EGF-R is oncogenic due to a mutation in its transmembrane domain (reviewed in Carpenter et.al., 1991). This mutation causes the neu proteins to aggregate with a concomitant increase in tyrosine kinase activity (Weiner et.al., 1989). The proto-oncogenic neu tyrosine kinase can be also stimulated by bivalent antibodies (Yarden, 1990) and EGF-R's and neu can form heterodimers (Goldman et.al., 1990).

9-Wild type and mutant EGF-R can heterodimerize with HER-2 in an EGF dependent manner, an effect tyrosine phosphorylation of proteins. If the EGF-R mutant lacks the cytoplasmic domain, however, heterodimerization occurs and tyrosine kinase activity is inhibited (Spivak-Kroizman, et.al., 1992). This implies that EGF dependent heterodimerization causes signals to be transduced depending on the exact nature of the components in the plasma membrane.

10-Dimerization of EGF-R monomers by EGF has been reported to be second order in receptor concentration as is the rate constant for this reaction and the detectable tyrosine kinase activity (Canalis, 1992). The author concludes that because of the fact that all the perameters change together, then dimerization of EGF-R and activation of tyrosine kinase activity are indistinguishable upon EGF addition.

Recently, EGF-R mutants have been created whereby the basic amino acids at the cytoplasmic face of the receptor have been either deleted or deleted and replaced with neutral amino acid residues (Yamane et.al., 1992). Removal of these residues in rodent fibroblasts had the effect of greatly inhibiting EGF dependent tyrosine phosphorylation of EGF-R. When EGF was added to these cells, the mutant receptor showed no morphological alterations as did cells containing the wild type receptor (Yamane et.al., 1992). The implication is, therefore that the basic residue in this domain of the receptor are very important in the transduction of signals by EGF-R, possibly through the topology of the receptor in the plasma membrane

In cell lysates, altering the lipid composition of the plasma membrane can modify ligand affinity and basal kinetic activity (Downward et.al., 1984), thus suggesting an active role for the transmenmbrane domain in signal transduction and confirming an intramolecular activation model for this process.

The question of EGF-R s tyrosine kinase activity, it's activation will be discussed in Chapter 5.

5.3.4.Summary.

The subject of EGF-R tyrosine kinase activation is a highly contentious one. Arguments for and against each model are strong. It is likely that future research will prove certain elements of each activation model to be correct, and so the accurate picture of how this receptor's tyrosine kinase activity is activated will really be a synthesis of each model.

5.4.The Protein Kinase Activity of EGF-R.

5.4.1.Introduction.

In addition to it's role as a growth factor receptor, EGF-R can act as an enzyme: phosphorylating tyrosine residues on a number of celluar proteins. This has been previously outlined. The tyrosine kinase activity of EGF-R is central to many aspects of it's functioning. EGF-R is also thought to be closely involved in the process of carcinogenesis-it's tyrosine kinase activity is believed to be pivotal in this process too.

A thorough understanding of this ability of EGF-R to phosphorylate tyrosine residues, and how this may be regulated is therefore vital to an understanding of how EGF-R works in normal circumstances and also how it may be involved in various pathological states.

The next few sections address the following points:

1-The tyrosine kinase activity of EGF-R.

2-The inhibition of protein kinase activity.

(N.B. For reviews on the equally as important dephosphorylation of proteins, see Alexander, 1990. Tonks and Charbonneau, 1989 and Hunter, 1989 deal with protein tyrosine phosphatases).

5.4.2.Protein kinases.

The phosphorylation of proteins (and the reverse reaction) is a vital intracellular regulatory mechanism. Protein kinases effect this regulation by phosphorylating certain amino acid residues: Ser, Thr or Tyr in target proteins.

The first protein tyrosine kinase activities to be intensively studied were those of the viral transforming proeins: the acutely transforming retroviruses. In the next phase of research, attention was turned to the protein tyrosine kinase activity demonstrated to be associated with growth factor receptors.

5.4.3.Functioning of Receptor Tyrosine Kinases.

A-Receptor Tyrosinse Kinases and Cellular Siganlling.

Ligand binding causes:

1-Increase in ion and glucose uptake.

2-Stimulation of membrane kinases.

3-Pinocytosis.

4-Membrane rufffling.

5-Cytoskeletal alterations.

6-Morphological changes.

7-Glycolysis.

8-Polyamine synthesis.

9-Phosphoryalton of Ribosomal S6 protein.

10-Gene transcription, for example;-myc/fos after about 2 minutes.

-protein/RNA/DNA synthesis within 3-20 hours (Rosen et.al., 1983)

These plieotropic cellular responses result ultimately in cell cycle progression, DNA synthesis and cell replication. To do this, receptor tyrosine kinases of the third group need exposure to their cognate ligand for at least 30 mintues, and type 1 and type 2 receptor tyrosine kinases need exposure for a unbroken period of at least 8 hours. Receptor tyrosine kinases can be either inducers of COMPETENCE such as PDGF orinducers of PROGRESSION such as EGF, (Pledger et.al., 1973). For example, PDGF can be mitogenic in NIH 3T3 cells without other factors being present and can uniquely stimulate protein kinase C by increasing phosphoinositide hydrolysis (Habenicht et.al., 1981).

Tyrosine kinases can also cause RNA production for example of secreted proteins (Cooper et.al., 1985). Transcripts for c-fos and c-myc can be detected witin 5 minutes and 1-2 hours respectively which suggest that they may be involved in the

control of cellular proliferation. Different ligands induce these genes to different levels,for instance in all cell types, PDGF, FGF and PMA all induce c-myc (Greenberg et.al., 1984). In mouse fibroblasts, and EGF responsive cell lines, PDGF is more potent than EGF and insulin is ineffective at stimulating c-myc transcription (Mueller et.al., 1991).

B-Protein Tyrosine Kinases and Development.

i.Introduction.

It seems that receptor tyrosine kinases are involved in controlling cellular differentiation and cell-to-cell interactions, especially during embryogenesis (Pawson and Bernstein, 1990). Evidence for this includes:

1-Receptor tyrosine kinase genes are highly evolutionarily conserved (reviewed in Pawson and Bernstein, 1990; and Sibley, et.al., 1988).

2-Receptor Tyrosine Kinases and their ligands are expressed in vertebrate embryogenesis (Pawson and Bernstein, 1990).

3-Receptor Tyrosine Kinases are expressed in specific cell lines, which implies that they mediate lineage specific functions (Pawson and Bernstein, 1990).

Development is a two fold process which requires the restriction of totipotency and the acquisition of the abiity to differentiate down specific lineages. These processes depend on changes in gene transcription and signal generation. This is the area in which tyrosine kinases are believed to exert some considerable influence: by transducing signals which determine cell fate.

To study this process, mutation of the receptor tyrosine kinase or the ligand or the downstream target(s) for the kinase, has been carried out, leading to the establishment of developmental mutants of a number of different species. The developmental mutants are equivalent to and depend upon changes in the sending, receiving and response to positional cues mediated by receptor tyrosine kinase signal transduction pathways.

To summarise then, receptor tyrosine kinases regulate cellular differentiation in response to local and environmental signals.

i.i.The Involvement of EGF-R Tyrosine Kinase In Ontogenesis.

Ontogeny is the history of the development of an individual organism. It is believed that tyrosine kinases are involved in this process.

In the Ovine foetal lung, EGF-R has been demonstrated immunohistochemically, from 51 days of gestation, in the epithelia of the conducting airways (Johnson et.al., 1989 (b)). As gestation progresses, receptor levels increase then plateau, with the highest levels being found in the epithelium of the peribronchial glands.

Late in development of the Ovine lung, EGF is beleived to have a number of important effects for example, it causes proliferation of epithelium of the trachea and bronchus (Johnson et.al., 1989 (a) and (b)).

To mediate these effects, EGF-R kinase activity is thought to phosphorylate a number of different cellular substrates such as lipocortin-1. In a study carried out by Johnson et.al., lipocortin-1 and EGF-R immunoreactive sites co-localize ,and the authors conclude that EGF-R acts through lipocortin-1 (Johnson et.al., 1989 (b)).

Some supporting evidence for this hypothesis is provided by similar work carried out in human lungs which showed immunohistochemically that EGF-R and lipocortin-1 are both present within the epithelium which lines the conducting tubules of human foetal lungs (Johnson et.al.,1989 (a) and (b)). Although this suggests a role for either or both proteins in lung development for example, due to phosphorylation of lipocortin-1 by EGF-R, it is at present not known how this might be brought about.

5.5.The Tyrosine Kinase Activity of EGF-R.

5.5.1.Introduction.

EGF-R has intrinsic tyrosine kinase activity (Cohen et.al., 1980; Schlessinger et.al., 1978). The enzymatic activity depends on ligand binding (Prywes et.al. 1986), and in intact cells EGF-R is constitutive phosphorylated upon a number of ser, thr and tyr residues. The purpose of this section is firstly to look at the phosphorylation ability of EGF-R in terms of ability to phosphorylate both itself and other proteins. Secondly to examine factors which are thought to regulate this activity and finally to discuss the consequences to the cell and the organism as a whole of this kinase activity.

5.5.2.Autophosphorylation of EGF-R.

A-The Process of EGF-R Autophosphorylation.

Autophosphorylation of EGF-R occurs in response to EGF (Carpenter, 1987 and King et.al., 1980).

Autophosphorylation of any single tyrosine residue in EGF-R is not vital for EGF-R signal transduction. Clark et.al. showed that in EGF-R-/- CHO cells, signalling and mitogenesis is unchanged if this single tyrosine phosphorylation reaction is prevented (Clark et.al., 1988). In addition, EGF can still bind and autophosphorylation can still take place but on the remaining tyrosine residues (Clark et.al., 1988).

Honegger's group carried out a series of experiments similar to those performed by Clark's group: they altered the autophosphorylation sites individually by site directed mutagenesis, converting the tyrosine to phenylalanine residues and transfected them into EGF-R -/- NIH 3T3 cells (Honneger et.al., 1988).

The results were that if one tyrosine residue is altered, EGF-R are still expressed at the plasma membranes. The receptors thus expressed are are of both the low and high affinity types, and they are responsive to PMA.

The receptors have EGF dependent tyrosine kinase activity: both autophosphorylation and phosphorylation of exogenous substrates. The receptors allow normal internalization and degradation patterns. Finally, the receptors are mitognic to EGF, but at a lower concentration of EGF than that required for mitogenesis by normal receptors.

So, except for the fact that the mutant EGF-R's are more sensitive to EGF than normal receptors, allowing or preventing autophosphorylation at individual tyrosines has no real effect upon EGF-R signal transduction (Honneger et.al., 1988).

This is consistent with the idea that autophosphorylation acts as a positive feedback inhibition process; i.e., it reduces the Km of kinase substrates which increases kinase activity at low substrate concentrations-the mutation derepresses this function. The finding that the mutated receptor has increased mitogenicity at low EGF concentration suggests that autophosphorylation establishes a stimulation threshold which must be exceeded to induce a mitognic response (Honegger et.al., 1988).

In terms of autophosphorylation and tyrosine kinase activity, the creation of 3' co-terminal mutations identified in addition the 3 autophosphorylated Y residues listed above, a fourth residue: Y992 which is autophosphorylated (Walton et.al., 1990). The mutated receptor show an increase in tyrosine kinase activity against exogenous substrates in vivo, except when Y992 is mutated. In this case, the result is a total abolition of all detectable EGF dependant tyrosine phosphorylation of proteins (Walton et.al., 1990). Such studies suggested that autophosphorylation could have an inhibitory function.

Gill et.al. suggest however, that autophosphorylation increases tyrosine kinase activity. Phosphorylation of EGF-R can be inhibited at high concentrations of exogenous peptide substrate. This effect can be relieved by initial autophosphorylation of EGF-R (Gill et.al.,1985). The suggestion is that autophosphorylation causes a loss of some type of constraint and this allows a substrate access to the active site of EGF-R.

How such effects might be brought about is not known. Y992 is located at the acidic, alpha-helical region of the protein. This region is vital for ligand induced receptor interaction, which suggests that Y992 may be involved in this process (Chen et.al., 1989). Two alternative models are that a non-phosphorylated EGF-R C-tail has affinity for the tyrosine kinase domain. Phosphorylation reduces this affinty, so the enzyme becomes available for interaction with substrates (Hsuan et.al., 1989). Finally, the C-tail may recognise substrates for tyrosine kinases (Pandiella et.al., 1988), for example phospholipase C and GAP protein (Schlessinger et.al., 1988).

Bertics and Gill showed that autophosphorylation caused an increase in kinase activity of EGF-R:Vmax was increased (Bertics and Gill, 1985). The ability to phosphorylate exogenous substrates was assayed as a function of the autophosphorylation state.

The resulting kinetics showed firstly that, at low ATP concentrations, the double reciprocal plot of tyrosine kinase activity against the amount of unphosphorylated EGF-R was hyperbolic i.e. ATP seems to activating the kinase. Secondly, at high ATP concentrations, autophosphorylation is increased and the kinetics are linear.

Exogenous substrates appear therefore to inhibit the receptor autophosphorylation reaction and the autophosphorylation sites therefore seem to be able to act as competitive inhibitors/alternative substrates against the peptide substrates. Bertics and Gill therefore concluded that autophosphorylation releases a competitive constraint, so that phosphorylation of exogenous substrates is facilitated (Bertics and Gill, 1985). Precedents for such a process are known: insulin receptor (Rosen et.al.,1983 and Yu and Czech,1984), Rouse Sarcoma Virus (Purchio et.al.,1983), Fujiama Sarcoma Virus (Weinmaster et.al., 1986) and erb-B (Gilmore et.al., 1985).

It would therefore seem that autophosphorylation sites compete wih exogenous substrates for the substrate binding region of the enzyme. Autophosphorylation releases an internal constraint so allowing the substrate access to the catalytic domain (Honneger et.al., 1988).

To complete this review of this very controversial topic, it should be stated that some investigators feel that autophosphorylation has no effect at all upon the tyrosine kinase activity of EGF-R (Downward et.al., 1984) on either purfied or plasma membrane bound EGF-R prepations (Cassel et.al., 1983).

MTLn is a metastatic rat mammary adenocarcinoma cell line (Lichtner et.al., 1992). EGF-R is found to be phosphorylated only in intact cells and it is though that the inhibition of this process is active(Lichtner et.al., 1992). Different forms of EGF-R were found to exist in this somewhat unusual cell line: 1 is 420-480 KD and the other is 95 KD, and the smaller protein is the one which is never phosphorylated (Lichtner et.al., 1992). Although the origins of these EGF-R types are unknown, their existence suggests that EGF-R signal transduction can proceed without receptor autophosphorylation.

5.6.The Phosphorylation of EGF-R.

5.6.1.General Details.

As well as EGF-R gene amplification, control of EGF-R gene expression and ligand binding, phosphorylation of EGF-R by a number of different agents can regulate its acitivty. Tyrosine, serine and threonine residues are phosphorylated which implies that there are a number of kinases involved, acting coordinately (Cochet et.al., 1988 (a) and (b)). This chapter will look at tyrosine phosphorylation, the phosphorylation of the other residues will be dealt will separately in Section 5.8.

When cultured cells are considered, EGF dependent tyrosine phosphorylation of EGF-R is observed to occur rapidly and is observed to be maintained for significant but variable periods of time: one hour in NIH 3T3 cells (Sturani et.al.,1990) and about six hours in A431 cells (Sturani et.al.,1988)i.e. this phenomenon is cell type specific. A small amount: approximately twenty percent of the total EGF-R population are tyrosine phosphorylated that is to say are active, in response to EGF (Carpenter et.al., 1987).

As in the case of liver regeneration, a number of different kinases are probably involved-but dephosphorylation reactions will also occur.

5.7. Tyrosine Phosphorylation of EGF-R by Exogenous Substrates.

The following table lists those agents or phenomena which are currently believed to cause the EGF-R to become tyrosine phosphorylated:

Table Three: Agents Which Tyrosine Phosphorylate EGF-R.

Agent	Reference(s).
EGF-Like Growth Factor	Higashijama et.al., 1992.
Hyper-Osmotic Shock	King et.al., 1985 and 1989.
Src	Wasilenko et.al., 1990.
Tumour Necrosis Factor	Donato et.al., 1989.
TGF-Alpha	Reynolds et.al., 1981.

5.8.Serine/Threonine Phosphorylation of EGF-R.

In addition to phosphotyrosine, EGF-R is phosphorylated upon serine and threonine residues (Downward et.al., 1984; Hunter and Cooper, 1987 and Carpenter, 1987). Such levels of phosphothreonine and phosphoserine increase upon treatment with EGF (Downward et.al., 1984).

The kinases responsible for these phosphorylation events are as yet unknown, as are to a large exetent, the consequenecs of these reactions. They do, however, appear to have an important role in the regulation of EGF-R functioning.

5.8.1.Threonine 654.

Treating intact 32P-labelled cells with phorbol esters, cause increased phosphorylation of serine and threonine residues (Hunter and Cooper, 1987 and Davis and Czech, 1985 (a),(b) and (c)). The same residues on EGF-R are phosphorylated by protein kinase C alone, as by phorbol esters in A431 cells (Cochet et.al., 1984 (a) and (b)).

T654 is located on the carboxyl face, nine amino acids from the plasma membrane. This location between the extracellular and cytoplasmic domains mean that it is ideally suited to effect the interaction between the two domains (Hunter and Cooper,1987).

Phosphorylation of T654 can occur via protein kinase C dependent and independent pathways and lead to loss of high affinity type receptors, reduced tyrosine kinase activity and transient internalization of receptors i.e. EGF-R down -regulation, (Downward et.al., 1984 and Beguinot et.al., 1985).

T654 is believed to mediate its effects by interfering with the interaction between the basic region of EGF-R and the phosphholipid heads in the plasma membrane (Hsuan et.al., 1989).

The cellular consequences of EGF-R tyrosine and serine phosphorylation are, as previously stated largely unknown. The theories that are presently held are also conflicting. Bowen's group maintain that phosphorylation of T654 in vitro leads to a reduction in tyrosine kinase activity of the receptor (confirmed by the findings of Cochet et.al., 1988 (a); Davis et.al., 1988 and Downward et.al., 1984).

Livneh et.al. treated NIH3T3 cells with phorbol esters and noted that T654 became phosphorylated and EGF-R signal transduction was inhibited (Livneh et.al., 1988). The constitutive overexpression of phosphorylated T654 in CHO cells (Livneh et.al., 1988) allowed the effect of phosphorylation of EGF-R in the absence of protein knase C activation to be studied separately for that phosphorylation of T654.

It was subsequently found that constiutive phosphorylation of EGF-R at T654 blocks mitogenic cellular signalling, implying that phosphorylation of EGF-R at T654 in intact cells causes inhibition of EGF stimulated cellular proliferation.

On the other hand though, evidence that T654 is not vital for negative regulation of the tyrosine kinase activity of EGF-R is provided by Friedman's group (Friedman et.al.,1989). These authors showed that the calcium ionophore A23187 and the non-PMA type phorbol ester Thapsigargin (Friedman et.al., 1989) can reduce the level of EGF binding and autophosphorylation in A431 cells, in a protein kinase C independent manner. T654 therefore is not the only site involved in the negative regulation of the enzymatic activity of EGF-R and EGF binding.

Activators of Protein kinase C phosphorylate EGF-R at T654 and this may also lead to the down regulation of EGF-R tyrosine kinase activity, for example in cytokine treated human gingival fibroblasts (Bird and Saklatval, 1989) cells exhbit reduction in EGF/EGF-R binding in a protein kinase C dependent manner where EGF-R numbers are reduced (Bird and Saklatvala, 1990). In addition to this effect, these agents cause the EGF-R to become Ser/Thr phosphorylated too and this process is not dependent upon protein kinase C which implies that T654 is not the only residue that is significant in this process.

5.8.2.Threonine 669.

This is a major site of phosphorylation of EGF-R (Countaway et.al., 1989 (a) and (b) and Heiserman and Gill, 1988). Close to T669 are two prolines. This site: -pro-leu-thr-pro- is phosphorylated by EGF, PDGF and phorbol esters. Phosphorylation might inhibit a protein phosphatase, mediate EGF-R transmembrane signalling leading to a change in the conformation of the receptor or activate a protein kinase. There is some evidence for this theory (Carpenter et.al., 1987) and some against it too (Davis et.al., 1988; Livneh et.al., 1988 and Cochet et.al., 1988 (a) and (b)).

T669 phosphorylation may regulate EGF-R internalization and tyrosine phosphorylation of exogenous substrates (Heiserman and Gill,1988 and Northwood et.al., 1991). Exactly how such a phosphorylation event might be carried out is not know though. The sequence outlined above is unusual and a rarely occuing phosphorylation site (Heiserman and Gill, 1988). Northwood et.al. propose that the following two kinases are responsible: MAP2 kinase and EGF-R Thr669(ERT)Kinase. ERT Kinase phosphorylates of T669 in the sequence -KRELVEPLT669PSGEAPNQALLR- (Downward et.al., 1984).

5.8.3.Phosphorylation at Serine and Threonine Residues.

Certain agents, listed in the table which follows, are known to phosphorylated the serine and threonine residues of EGF-R.

Table Four: Agents Which Mediate Ser/Thr Phosphorylation of EGF-R.

Agent	Reference
Ceramide	Hannun et.al., 1986; Okazaki et.al., 1987;
Protein Kinase C	Iwashita et.al., 1984 and 1990; Downward et.al., 1984; Davis et.al., 1988.
Phorbol Esters	Lee et.al., 1988 and 1989;
Okadaic Acid	Biajolan and Takai, 1988.
Shingosine	Davis et.al. 1985 (a), (b) and (c).

Table Five: Phosphorylation of EGF-R by Exogenous Agents.

Substrate	Reference
Enolase	Reiss et.al., 1986.
Erythrocyte Band 3	Shiba et.al., 1986.
Ezrin	Gould et.al., 1986 and 1988.
Gastrin	Baldwin et.al., 1983 (a) and (b).
Glucose-6-phosphate Dehydrogebase	Napier et.al., 1987.
Growth Hormone	Baldwin et.al., 1983 (a) and (b).
Integrin	Bellas et.al., 1991.
Lactate Dehydrogenase	Reiss et.al., 1986.
Lipocortin1	Abdel-Ghany et.al., 1989.
Microtubule Associated Protein Kinase 2	Takishima et.al., 1991 and Sano and Kitajima, 1992.

Middle T Antigen	Segawa and Ito, 1983.
Myosin Light Chain	Gallis et.al., 1983.
Phosphoglycerate Mutase	Reiss et.al., 1986.
Phospholipase C	Wahl et.al., 1987, 1988, 1989 (a) and (b).
Progesterone Receptor	Ghosh-Dastidar et.al., 1984.
Raf-1	Baccarini et.al., 1991.
Sodium/Hydrogen Exchanger	Sardent et.al., 1991.
Vinculin	Bellas et.al., 1991.

(N.B.: Some as yet unidentified cellular proteins have been found to be phosphorylated by EGF-R: in neural cells (Girault et.al., 1992) and in NIH 3T3cells which contain egf-r or egf/erb-B2 constructs, p97, p68, p67, p61, p56 and p23 (Fazioli et.al., 1992)).

5.9 .The Regulation of EGF-R Tyrosine Kinase Activity.

EGR-R kinase activity can be regulated by phosphorylation (auto-phosphorylation, tyrosine phosphorylation and serine and threonine phosphorylation), ligand binding and EGF-R metabolism events (internalization and degradation).

It is also subject to regulation by a number of heterologous stimuli in the process known as Transmodulation (Carpenter, 1987). Exactly how receptor transmodulation is brought about is not known, but it is most likely to be due to the interaction of different signal transduction pathways (Di Marco et.al., 1989 and Wong et.al., 1989 (a) and (b)). Protein kinase C probably has a large role to play too (Hicks et.al., 1989 and Northwood and Davis, 1988 and 1989).

Table Six:Agents known to Regulate the Tyrosine Kinase Activity of EGF-R.

Agent	Reference
cAMP/cAMP dependent kinase(Protein kinase A)	Iwashita et.al., 1984 and 1990; Rackoff et.al., 1984; Walker and Pike, 1987; Gill and Lazar, 1981;
EGF	Chinkers and Garber, 1986; Schaudies et.al., 1985.
Gangliosides	Song et.al., 1991; Feitzi and Childs, 1985.
4-Hydroxytamoxifen	Engel and Young, 1978; Wakeling et.al., 1989; Freiss et.al., 1990.
Mullarian Inhibiting Substance	Cigarroa et.al., 1989.
Neu	Wada et.al., 1990; Weiner et.al., 1989.
Phorbol Esters	Northwood and Davis,1988.

PDGF	Decker and Harris, 1989; Walker and Burgess, 1988; Wran et.al., 1980; Olashaw and Pledger, 1988.
Protein kinase C	Northwood and Davis, 1988 and 1989 and Schechter et.al., 1984.
Second Messengers	Verheijden et.al., 1990.
Src	Wilson et.al., 1985 and 1989; Kaplan et.al., 1988.
SH2-Domains	Anderson and Marchesi, 1985.
Sphingosine	Davis et.al., 1985 (a), (b) and (c) and 1988.
TGF-Alpha	DiMarco et.al., 1989; Wong et.al., 1989 (a) and (b).

5.10.The Biological Consequences of the Activation of EGF-R Tyrosine Kinase Activity.

5.10.1.Introduction.

The activation of the enzyme actvity of EGF-R has a number of biochemical effects which eventually culminate in the replication of the DNA and the cell itself (Gill et.al., 1985). Exactly how this kinase activity is involved in this process is not known.

It seems functional EGF-R tyrosine kinase activity is vital for all (or at least a majority)of the metabolic effects of EGF-R (Yarden and Ullrich, 1988). The task which now remains-is to identify the cellular elements which are located down-stream of this kinase.

What follows is an attempt to appraise the current understanding of the consequences for a cell and the organism as a whole of the activation of the kinase activity of EGF-R.

5.10.2.EGF-R Tyrosine Kinase Activity and EGF-R Signal Transduction.

EGF-R's catalytic function alone is not sufficient for signal transduction-carboxyl elements confer signal specificity. This is demonstrated by the sixty three amino acid C-terminal deletion mutant i.e. an EGF-E with no autphosphorylation sites. This receptor has no normal ionic signalling, insitol phosphate turn-over or DNA synthesis (Schlessinger et.al., 1978). This activity is however,essential for signalling. Some of the presently accepted evidence for this point of view is as follows.

The lys-721 forms a crucial part of the ATP binding domain. Mutation from L to M here and the transfection of this construct into EGF-R -/- mouse L cells (B82) and CHO cells (Chen et.al., 1987) had the following effects:

-mutant EGF-R is expressed an wild type levels.

-mutant EGF-R binds EGF-R with wild type affinity.

-mutant EGF-R is 170 K.D.

-mutant EGF-R has no intrinsic tyrosine kinase activity whatsoever. There is no evidence of ligand

dependent-endocytosis.

-gene transcription.

-cell replication.

-intracellular calcium increase.

A L721AcDNA contruct transfected into NIH3T3 cells (Honnegger et.al., 1988) had the following effects: no tyrosine kinase activity, normal receptor processing and normal expression of the receptor at the plasma membrane. In addition, the receptor cannot stimulate early responses (such as Na+/H+ transporter activation, calcium signalling and inositol phosphate formation) and late responses (like DNA synthesis, cell replication, focus formation and gene expression: c-fos and c-myc).

This suggests that the tyrosine kinase activity is vital for EGF-R signal transduction and certain biolgical effects.

Premature termination mutants of EGF-R lacking the C-terminal and kinase domain (Velu et.al., 1980, 1989 and 1990) have reduced biological activity but again the number of receptors and early events were in the wild type range. This suggested that the C-domain is involved in the regulation of EGF-R tyrosine kinase activity, in a positive sense.

The N-terminal region also has a vital part to play in this process. Consider the example of v-erb-B, where N-terminal truncation has resulted in the oncogenic activation of the protein

(see Downward et.al., 1984 and Maihle and Kung, 1988). The constitutively active, EGF independent receptor that is thus generated (Raines et.al., 1988 and Wells et.al., 1988) renders the gene capable of inducing erythroblastomas.

5.10.3.Phosphatidyl Inositol Metabolism.

In A431 cells, EGF causes the breakdown of polyphosphoinositides, the production of inositol phosphate and also an increase in PIP levels which implies the activation of phosphatidylionsitol kinase too (Pike and Eakes, 1988).

Cholera toxin selectivley inhibits the second of the effects noted above but phosphatidyl inositol breakdown remains unaffected by it. Pertussis toxin affects neither process (Pikes and Eakes, 1987). This subject is discussed in Section 5.12.

5.10.4.Phospholipase C Regulation.

EGF causes phospholipase C-Y to be activated, in a process which is dependent upon the tyrosine kinase activity of EGF-R (Moolenaar et.al., 1988). If pure EGF (and PDGF) and phospholipase C-Y are incubated together then phospholipase C-Y becomes phosphorylated-this occurs at physiological concentrations of EGF (Meisenhelder et.al., 1989 and Nishibe et.al.,

1990 (a) and (b)). The phosphorylations take place on particular tyrosine residues of phospholipase C-Y, which has suggested to the above authors that this is the signal which effects growth factor signal transduction.

These findings have been supported by other work which has shown that EGF enhanced EGF-R/phospholipase C-Y association in an EGF dependent manner (Margolis et.al., 1989 (a) and (b)and 1990). This work was taken a little further in that it was shown that if tyrosine kinase defective EGF-R's were used this association was not seen (Margolis et.al., 1989 (a) and (b)and 1990). The inference was made that the tyrosine kinase activity of EGF-R is vital to allow the phosphorylation of phospholipase C-Y.

The current hypothesis is that the association between these two proteins forms the equivalent of an enzyme/substrate intermediate.

5.0 .5.G-Protein Phosphorylation.

Do EGF-R's associate with G-proteins and so transduce EGF signals?. Again this is a highly controversial area. EGF-R does phosphorylate a 22-25K.D protein (Gp)which has high affinity (Kd=50-100nm) GTP binding (see Section 5.15.). This work used

phospholipid vesicles reconstituted with pure EGF-R tyrosine kinase domain and a number of different GTP binidng proteins to see if the polypeptides could be a substrate for the kinase.

The results indicated that indeed tyrosine residues within this protein were phosphorylated-they were the only residue to be phosphorylated. Importantly, phosphorylation depended upon the insertion of the G-protein into the lipid bilayer-raising the suspicion that the phospholipids of the membrane in some way help to orient the kinase and substrate in an optimal manner.

GTP and GTPYS inhibit this phosphorylation reaction implying that the G-nucleotide occupancy is being affected. It could possibly alter the 22K.D. protein's conformation so reducing the chance that it may be phosphorylated.

Reconstituting EGF-R and Gp in platelets allows EGF dependent phosphorylation of Gp, and this process is sensitive to the addition of GDP and GTP analogues.

The theory is therefore that tyrosine phosphorylation of the Gp causes it to be released from the plasma membrane. This allows it to react with cytoplasmic effectors.

5.10.6.Cellular Proliferation.

EGF has been known for many years to stimulate prolferation of cells (Carpenter, 1987), but how this might be brought about is not known. It seems that intact tyrosine kinase domains are vital for this process though (Chen et.al., 1987), since the EGF-R mutant K721M cannot effect DNA replication and cell division (Chen et.al., 1987).

In addition, EGF dependent transcription of specific genes is prevented and this may provide an important clue as to how EGF-R tyrosine kinase may be mediating this effect, for example, transcription of c-jun (Quantin and Breathnach, 1988) is inhibited. These genes encode transcription factors (Curran et.al., 1982) i.e. they orchestrate the transcription of other genes. Since their expression is initiated very early after exposure to EGF (Carpeneter,1987 and Gill et.al., 1985)this implies that if the are not expressed or are expressed aberrantly in some way, the cascade of gene expression for which they are responsible (and which cumulates in mitogenesis) will not be activated or will follow different lines.

EGF has been regarded as a progression factor (see Section 5.4.9-A). These are agents which act after an initiating factor to cause mitogenesis-but which cannot induce mitogenesis alone. Some research has now suggested that the quantity of the signal generated by EGF-R which is dependent upon the number of receptors present on the plasma membrane is the crucial feature which allows proliferation to be stimulated (Velu et.al.,1989). This concept of a stimulation threshold is borne out by the fact that in order for mitogenesis to occur, EGF/EGF-R must be in contact for a significant period of time.

That the tyrosine kinase activity of EGF-R has a prime role here is hinted at by the following pieces of evidence. Firstly the L721A mutant version of the EGF-R cannot induce transcription of c-fos or c-myc; the morphological changes seen normally and DNA synthesis (Honegger et.al., 1987 (a) and (b) and 1988). An intact tyrosine kinase domain is essential for receptor tyrosine kinases of type I and III to have transforming ability (Roussel et.al., 1987). This effect is enhanced by mutating the N- and C-termini (Roussel et.al., 1987).

Prevention of cytoplasmic acidification, through protein kinase C activation of the Na+/H+ exchanger is vital for cell growth (Pouyssegur et.al., 1985).

5.10.7.EGF-R Tyrosine Kinase and EGF-R Metabolism.

A.Introduction.

Receptors often show down-regulation in response to ligand binding (Gill et.al., 1985). In the case of EGF-R, the following sequence of events occurs (Schlessinger, 1986):

1-In the resting state, EGF-R are diffuse on the plasma membrane of the cell (Schlessinger, 1986).

2-EGF and EGF-R bind, in a temperature dependent manner.

3-Receptor/ligand complexes exhibit rapid lateral mobilty.

4-Receptor/ligand complexes cluster in coated pits (Beguinot et.al., 1984 and 1985)

5-Complexes are internalized, formimg clathrin-coated vesicles (Haigler et.al., 1978; Schlessinger et.al., 1978; Carpentier et.al., 1986 and 1987 and Hertel et.al., 1987).

6-Vesicles are uncoated.

7-Vesicles are acidified, forming endosomes.

8-Endosmes are sorted: they can be either recycled to the plasma membrane or are broken down in the lysosome (Goldstein et.al., 1985 ; Carpenter and Cohen, 1990; and Beguinot et.al., 1984 and 1985).

The events of EGF-R metabolism are therefore clearly important to the functioning of EGF-R as a receptor for a ligand, as an enzyme and as a signalling molecule. Much evidence states that the tyrosine kinase activity of the receptor is vital for these various activities to take place. This will be discussed in the sections which follow.

B-EGF-R Internalization.

Biochemical evidence for EGF-R internalization was first provided by Carpenter and Cohen in1976 (Carpenter, 1987). They showed ligand/receptor internalization by determination of the course of bindning of 125-I EGF to human fibroblasts at 37 and 0 degrees centigrade. Under these conditions, maximal binding occured at 45-60 minutes. At 37 degrees centigrade, however, if the labelled ligand and cells were incubated further, the amount of cell-bound radioactivity decreasd to a level of 20-25% maximal. At O degrees centigrade there was no loss of cell bound radioactivity.

It was therefore postulated that after initial binding, the ligand/receptor complex is internalized and degraded intracellularly since cell bound labelled EGF was rapidly degraded to mono 125I-iodotyrosine at 37 degrees centigrade. At 0 degees centigrade, cell-bound 125-I EGF was not degarded, but slowly dissociated from the cell.

At 37 degrees centigrade for 125 I EGF bindigng , followed by incubation at 0 degrees centigrade, almost no release of cell bound radioactivity could be detected. Degradation, but not binding requires metabolic energy. Degradation is inhibited by drugs which inhibit lysosomal functioning: chloroquine and ammonium chloride.

When 125I EGF was bound to cells at O degrees C, the ligand was much more accessible to surface reactive agents such as trypsin and EGF-Antibodies than at 37 degrees centigrade. Exposure of fibroblasts to EGF resulted in an apparent loss of plasma membrane receptors for EGF implying that the receptors are internalized too.

EGF-R tyrosine kinase activity is thought by many to be essential for receptor internalization to take place. Some of the evidence supporting this point of view is as follows. Firstly, L721M: an EGF-R mutant (Chen et.al., 1987)which has no tyrosine kinase activity shows no receptor internalization.

In addition, immunofluorescent staining using anti-phosphotyrosine antibodies to reveal the proteins which have been tyrosine phosphorylated in response to EGF showed that tyrosine kinase active EGF-Rs are distributed evenly on the cell surface, and are endocytosed when EGF is added. EGF-R with no tyrosine kinase activity although are distributed evenly upon the surface of a cell in the absence of EGF when EGF is added, they remain on the surface (Glenny et.al., 1987 (a) and (b)).

Studies by (Chen et.al., 1987; Hari and Roth, 1987; Honegger et.al.,1988 and 1989 and Russell et.al, 1987) using tyrosine kinase negative EGF-R showed ligand dependent internalization did not occur. Gill proposes that tyrosine

phosphorylation mediated this effect by routing the receptor into a pathway which concludes with receptor mediated endocytosis or receptor recycling.

The kinase specificity of chimaeric EGF-R are determined by elements within the cytoplasmic domain. These determinants also affect the fate of the EGF-R after it is internalized (Riedel et.al., 1988 and 1989).

Finally, site directed mutagenesis of T669 and S671 residues, sites of phosphorylation in EGF-R showed that they were involved in ligand dependent endocytosis (Heiserman et.al., 1988).

These observations are consistent with the need for phosphorylation of a cellular substrate(s) which is involved in endocytosis and autophosphorylation. This is a central tenent of the intramolecular model for the activation of EGF-R tyrosine kinase activity (See section 5.3.2.).

To summarise then, substrates vital for endocytosis need to be appropriately phosphorylated; autophosphorylation may be needed to allow EGF-R/endocytotic vesicle interaction or both autophosphorylation and phopshorylation of a cellular substate are needed for endocytosis.

The situation may not be quite so straight forward though as there are reports that tyrosine kinase activity is not needed for EGF-R internalization. Honneger and Felder found that a tyrosine kinase defective EGF-R can still exhibit EGF dependent internalization (Honneger et.al., 1988, 1989 and 1990 and Felder et.al., 1990).

Using antibodies to prevent phosphorylation of EGF-R, shows that in the unphosphorylated state, the receptor can internalize in response to EGF (Sunada et.al., 1986) implying that the phosphorylation state of the receptor is not important.

However, abolishing the kinase activity associated with EGF-R by monoclonal antiphsophotyrosine antibodies preveted ligand induced receptor internalization (Glenney et.al., 1987 (a) and (b))

WB-344 cells are non-transformed epithelial cells (Tsao et.al., 1984). They were used as a model for this process of EGF-R internalization and it was found that there was at least four stages through which EGF-R tyrosine kinase passes upon EGF binding (McCune and Earp, 1989).

Firstly there is kinase activation. This occurs within seconds of EGF binding and is responsible for the main tyrosine phosphorylation of EGF-R and it's internalization. A receptor

conformational change then occurs. Autophosphorylation and tyrosine kinase activities are reduced. All this occurs within about 2-5 minutes of exposure.

Firstly kinase activity then becomes re-established. This takes place 10-30 minutes afer internalization and may depend on sequestration by EGF-R from cellular ATP pools as it moves into intracelluar vesicles. Finally, transfer of EGF-R to lysosomes takes place. This results in the total abolition of tyrosine kinase activity and may depend upon EGF-R degradation in the lysosomes.

The current theories for the suppression of EGF-R autophosphorylation activity are that a serine/threonine protein kinase may be activated. Very high EGF concentrations will be required though to mediate this effect, which suggests that EGF-R occupancy may be the important feature:the kinase is activated only if large numbers of EGF-R are occupied with ligand.

A conformational change in the EGF-R molecule could take place which reduces autophosphorylation activity, such as dimerization of receptors. Recent evidence sugests that the acidic environment of the endosome causes conformational changes in EGF-R (DiPaolo and Maxfeld, 1984).

An association of EGF-R and cytoskeletal elements is also possible (for further details, see Kwiatkowska et.al., 1991 and Van Bergen en Henegouwen et.al., 1989). The cytoskeleton therefore inhibits EGF-R tyrosine kinase domain movement so reducing it's activity. The endocytic pathway/network that is established as a result is most dense at the plasma membrane.

The work of Chen has identified a putative EGF-R Internalization Domain (Chen et.al., 1987). It seem that the carboxyl domain has inhibitory elements for kinase activity and that they are active in the non-phosphorylated state. Autophosphorylation or phosphorylation by an exogenous agent causes the release of the inhibition to take place exposing the internalisation domain.

Removal of the C-terminal 174 amino acids allows the partial rescue of the tyrosine kinase -/- EGF-R internalization ability (Chen et.al., 1987 and later in this section).

It is not known if, when internalized, EGF-Rs are in a functional or a non-functional state, that is to say can they phosphorylate proteins?. This is a very important question since the tyrosine kinase activity of EGF-R is believed to be involved in a number of different intracellular processes. There is, however little actual evidence to support or refute such a point of view.

The Role of the Carboxyl Domain in Internalization of EGF-R.

Sequences here seem to be essential for EGF-R internalization. C-terminal truncation of the tyrosine kinase domain created receptor which cannot mediate ligand dependent down-regulation (Gill et.al., 1985). A 44 amino acid truncation of EGF-R cannot undergo ligand dependent down-regualtion (Livneh et.al., 1986 (a) and (b) and 1987). In addition, EGF does not induce internalization of an extracellular domain egf-r/v-abl tyrosine kinase domain chimaeric receptor (Prywes et.al., 1986).

A series of 3'-terminal detetions of egf-r showed that the distal boundary of the tyrosine kinase domain is at residue 957 (Chen et.al., 1987). This is at an exon/intron junction (Haley et.al., 1987).

The 229 amino acids C-distal to residue number 957 cause EGF dependent increase in calcium levels, receptor endocytosis and receptor down-regulation (Chen et.al., 1987). The 48 amino acids located between residues 973 and 1022 determine calcium regulation and receptor endocytosis and have been named the "Calcium Internalization Domain" or "CALN".

Permanent cell lines exressing EGF-Rs with no CALN domains have been set up to allow it to be analysed. These studies showed that EGF induced EGF-R endocytosis and calcium

mobilization are separate events with the later dependent upon EGF-R tyrosine residue autophosphorylation. The autophosphoryktion of the C-terminus of EGF-R has been found to mediate EGF-R/phospholipase-C interaction via SH2 domains. The existance of this domain could therefore possibly couple the pathways of EGF-R tyrosine kinase activation to that of calcium signalling.

The 164 amino acids distal to the CALN domain have also been found to constitute an inhibitory region (Chen et.al., 1987). The removal of these residues allows the internalization of kinase negative EGF-Rs via a low affintiy, high capacity pathway.

Residues number 973 to 991 are vital for internalization and calcium increases due to a cellular redistribution of intracellular calcium and the opening of calcium channels in the plasma membranes of the cell (Chen et.al., 1987). The 18 amino acids of the CALN domain form a helical structure, bound by turns and within this region are contained 9 acidic amino acids. This structure greatly resembles that of GAL4 and GCN4-yeast transcription factors (Ptashne et.al., 1986). These protein factors:i.e. their acidic helical motif sections act to couple enhancers to the transcription apparatus. The suggestion therefore is that this CALN domain couples ligand activated EGF-Rs to the calcium regulation and receptor internalization apparatus.

The Function of EGF-R Internalization.

Internalization of EGF-R is a well documented yet controversial area. What the exact effects upon the cell are is also a contentious issue. Internalization and processing of EGF-R may be important for signal transduction and mitogenesis (Wakskull and Wharton,1985 and Yanker and Shooter, 1982). For example, it's inhibition leads to inhibition of mitogenesis (Wakskull and Wharton, 1985). Inhibiting processing also causes inhibition of EGF dependent mitogenesis (King et.al., 1989 (a), (b) and (c)).

C.Intracellular Sorting of EGF-R and Tyrosine Kinase Activity.

Endocytosis via coated pits can occur in a number of ways. In the first way receptor and ligand are both recycled, for example the transferrin receptor (Goldstein et.al., 1985). A second way involves the receptor recycling, and the ligand being degraded, for example, the Low Density Lipoprotein receptor and insulin receptor (Goldstein et.al., 1985).

Finally, both receptor and ligand can be degraded, this is the case for the EGF-R (Goldstein et.al., 1985). EGF-R, however, depending on the cellular circumstances, seem to be able to undergo sorting by any one of these methods (Honegger et.al., 1987 (a) and (b)).

Receptor mediated endocytosis is a second-order process i.e., receptors compete for access to binding sites (Wiley et.al., 1985 and Lund et.al.,1990). This implies that the amount of receptor occupancy influences the fraction of receptors which enters the cell to be degraded.

Empty and full receptors, are however recycled and endocytosed at the same rate. Kinase negative EGF-R when EGF binds have an unchanged internalization rate and EGF-R with normal kinase domains have an elevated internalization rate (Wiley et.al., 1985). The suggestion therefore is that ligand induced down-regulation is mediated via changes in the rate of endocytosis, not the rate of recycling.

Occupancy induced endocytosis causes EGF dependent EGF-R down-regulation. This in some way targets the occupied receptor to the lysosome which results ultimately in a reduction in receptor numbers at the plasma membrane and an increase in the number of receptor in the internal pool (Wiley et.al., 1985).

Lysosomal targeting seems to depend upon the location of the receptor with in the Multi Vesicular Body or MVB (Felder et.al.,1990). The tyrosine kinase activity may therefore act to stabilise receptor/receptor interactions in coated pits or at sites further down the internalization pathway. The phosphorylation of additional proteins may also be involved in this process.

The post-internalization trafficking pattern of EGF-R seems also to depend upon the cell type. For example, in transfected B82 cells (Dunn et.al., 1986 and 1984); Human fibroblasts (Stoscheck and Carpeneter, 1984 (a) and (b)). Consider also the situation in polarized cells such as hepatocytes (Maratos-Flier, 1987). These cells internalize EGF only at their sinusoidal and lateral surfaces (Dunn et.al.,1986).

In Madin-Derby Canine Kidney cells too, EGF is transported from the basal to the apical surface of the cell (Brandi et.al., 1991). How this directionality might be brought about and it's importance, in terms of functioning are not known.

The role of the intrinsic tyrosine kinase activity of EGF-R in the process of intracellular trafficking of EGF-R is controversial with a number of groups stating that tyrosine phosphoryaltion of proteins is vital for sorting and others believing that it is not.

Some evidence that the kinase activity of EGF-R is not involved in intracellular sorting, is as follows. The K721A EGF-R mutant (Honegger et.al.,1987), the K721/T654A double EGF-R mutant (Glenney et.al., 1988 (a) and (b)) and the L721M mutant EGF-R (Chen et.al.,1989), all are kinase negative but with the exception of the first case they are not internalized.

K721A, via radiolabelling and ultrastructural analysis was found to internalize EGF-R, but these receptors are not degraded but are mainly recycled to the plasma membrane (Felder et.al., 1990). Initially, normal EGF-R and K721A type EGF-R internalization pathways are that same. The diverge at the MVB within the cell. This suggests that tyrosine phosphorylation here in the MVB generates a sorting signal which causes normal EGF-R to be degraded (Felder et.al., 1990). The sorting of EGF-R within the cell therefore depends upon spatial segregation in the MVB which in turn depends upon phosphotyrosine status.

The tyrosine kinase activity of EGF-R in some way seems to provide a sorting signal during receptor endocytosis which allows selection of EGF-Rs which are destined for degradation in the lysosome.

D.EGF-R Recycling.

The capacity of EGF-R to enter the degradative pathway could conceivably therefore depend on the tyrosine kinase activity of the receptor (Beguinot et.al., 1984 and 1985). There is no evidence for a non-lysosomal/recycling pathway in cultured fibroblasts (Beguinot et.al., 1984 and 1985) and KB cells (Lyall et.al., 1989).

In the following cell lines however, EGF-R do seem to recycle: pancreatic carcinoma cells (Korc et.al., 1986 and 1987); 3T3 fibroblasts (Wakshull and Wharton,1985) and hepatocytes (Dunn et.al., 1986 and Gladhaug et.al., 1988).

Sorting therefore seems to depend on structural features of the receptor (Riedel et.al., 1988 and 1989) and on cellular elements. What these might be are unknown, but mannose phosphate residues found upon EGF-R (Todderud and Carpeneter, 1986) may be important. For a lysosomal enzyme to sort to the lysosome, mannose phosphate is vital in allowing the appropriate enzymes to interact with with the mannosyl receptor and so allow these proteins to be separated out from secreted proteins (Von Figura and Haslik, 1986).

E.The Degradation of EGF-R.

Degradation is the final stage in EGF-R metabolism. The details of this process, like the ones preceeding it are unclear. The situation is further complicated by the fact that different cells regulate degradation using different mechanisms (Gladhaug, et.al., 1988 and Felder et.al., 1990).

There is a growing body of evidence which suggests that the intrinsic tyrosine kinase activity of EGF-R is essential for EGF dependent degradation. Kinase negative EGF-R mutants recycle but are not degraded in the presence of EGF (Glenny et.al., 1987 (a), (b) and (c) and Honegger et.al., 1988 (a) and (b)). Exactly what the tyrosine kinase does is, however, unclear.

Linked to this are studies involving site directed mutagenesis of Lys 721 in the ATP binding domain of the receptor. This alteration results in the creation of an EGF-R which has in addition to no kinase activity (Glenny et.al., 1987 (a), (b) and (c) and Honegger et.al., 1988 (a) and (b))an increased half-life. This may be due to either a reduced rate of receptor endocytosis (Glenney et.al., 1987 (a), (b) and (c)).

It must also be considered that other kinases can phosphorylate EGF-R too. Phorbol esters, for example, induce phosphorylation of EGF-R at T654 (Cochet et.al., 1988 (a) and (b)and Hunter et.al., 1984 and 1991). Phorbol esters also cause EGF-Rs to be internalized without being degraded (Beguinot et.al., 1984 and 1985). The role of T669 and S671 phosphorylations are unclear.

Degradation takes place principally in the lysosome (Velu et.al., 1989), but also in endocytic vesicles (Diment and Stahl, 1985; Korc et.al., 1987 and Yanagishuta et.al., 1985). Receptors have been located within the limiting membrane of endocytic vesicles and inside the MVB of KB cells too (Beguinot et.al., 1984 and 1985). Proteolytic cleavage of EGF-R in addition reveals that the N-terminus is mainly oriented in the center of the endocytic vesicle and so is exposed to intravesicular proteases (Decker, 1989). Degradation can be inhibited by low temperature treatments which inhibits internalization (Carpenter,1987).

The extracellular domain of EGF-R in hepatocytes is degraded in the lysosomes with a half-life of approximately 0.5-9 hours at 37 degrees C (Beguinot et.al., 1984). The tyrosine kinase domain is degraded along with the extracellular domain in lysosomes, and is not transferred to the cytosol or nucleus i.e. it does not serve as a second messenger (Beguinot et.al., 1984).

Cytoplasmic domains have to be degraded after EGF-R are sequestered into endosomes, but it is not known where this may occur, what the topology of the internalized EGF-Rs might be, or if degradation depends on cytosolic proteases and/or acid hydrolases (Renfrew and Hubbard, 1991).

Carpenter and Cohen showed that in fibroblats, EGF binding led to the degradation of EGF-R (Carpenter and Cohen, 1990). This was later confirmed by a number of other studies involving the use of anti-EGF-R monoclonal antibodies (Beguinot et.al, 1984 and 1985). Morphological analysis also showed that receptor and ligand are degraded together since both are found in MVBs (Haigler et.al., 1978 and McKanna et.al., 1979)

Recycling, on the other hand may be a mechanism which acts to amplify signals issuing from EGF-R. Such a model could explain the differential effects of EGF and TGF-alpha upon various celluar processes: TGF-alpha is a stronger agonist in the cases of calcium release from cultured bone cells (Ibbotson et.al., 1986) and stimulation of arterial blood flow (Gan and Hollenberg, 1989). TGF-alpha may be more rapidly degraded and/or degraded to a greater extent than EGF

5.11.EGF-R Signal Transduction and Ionic Fluxes.

5.11.1.Sodium/Hydrogen (Na+/H+)Exchange and EGF-R Signalling.

Na+/H+ exchange is the best known of the ionic transport changes stimulated by growth factor treatment of cells i.e. the growth factor activated a usually quiescent Na+/H+ exchanger located in the plasma membrane. Early evidence of the importance of Na+/H+ exchange was provided by work in neuroblastoma cells.

Addition of serum caused the activation of an electrically silent sodium influx pathway. This pathway is Amiliroide sensitive and can be stimulated with weak acid treatment of the cytoplasm (Moolenaar et.al.,1981, (a) and (b))this uptake of sodium is coupled to the efflux of hydrogen ions from the cell in a 1:1 stoicheometry (Moolenaar et.al.,1981 (a) and (b)).

The model therefore is that mitogenesis causes an increase in intracellular pH of 0.2-0.3 units and sodium entry which causes the Na+/H+ pump to be activated (Moolenaar et.al., 1881 (a) and (b)). Most cells maintain their pH at 7.0-7.4, which is well above the electrochemical equilibrum value of 6.0-6.4 predicted from transmembrane potential of approximately 60mV by the Nernst equation. In vertebrates, a specific hydrogen

extruding mechanism raises intracellualr pH (Moolenaar et.al.,1986 (a) and (b)). At normal pHi values the exchanger is relatively inactive but if pHi falls below a threshold level, the exchanger is increasingly activated.

Receptor occupancy may lead to an alkaline shift in pHi sensitivity to the exchanger (Moolernaar et.al.,1983; Paris and Poussegur et.al.,1984 and Grinstein et.al,. 1985). This altered pHi sensitivity may in turn be due to a conformational change in the exchanger protein which results in an increased pKa of the regulatory hydrogen binding site. The physiological effects of a growth factor upon the Na+/H+ exchanger therefore seem to be to increase it's pHi threshold: i.e. the level to which pHi must rise before the exchanger is shut off.

Growth factors are thought to modify pHi sensitivity of the exchnger and to raise pHi in the following way. Both TPA and synthetic DAG can mimick the effects of growth factors on the exchanger and pHi in various cell types implying that protein kinase C could directly phosphorylate the exchanger protein. This activation could be indirect though.

This putative involvement of protein kinase C suggests that an alkaline shift in pHi is not induced uniquely by growth factors, but is a common response in the action of those hormones and neurotransmitters that trigger the hydrolysis of

inositol lipids. For example, in hormone induced inositol phosphate turnover, in neutrophils, pH increases after activation by physiological stimuli (Molski et.al.,1980).

It seems then that pHi as regulated by the Na+/H+ exchanger plays a vital role in a number of cellular processes. Growth factors activates this antiporter in quiescent cells by altering its pH sensor so that the cytoplasm becomes more alkaline.

5.11.2.EGF-R and Calcium Levels.

Calcium was among the first intracellular messengers found in cells and is a crucial activator in many cells. It often acts along with other mesengers to modulate calcium fluxes themselves (Rasmussen et.al.,1989). Calcium enters cells through voltage operated channels or VOC's (Darnell et.al., 1986). VOCs are modulable by cAMP, protein kinase C, calcium and G-proteins (Tsien et.al., 1988).

Calcium channel activity is dependent upon voltage. They have the properties of single channels and pharmacological analyses showed that there are two types (Fox et.al.,1987). There are T-type channels which have a low threshold and have rapid effects (Rink and Merritt, 1990). L-type channels on the other hand have a high threshold and mediate slow effects (Tanabe et.al., 1987).

More recently, a third type of channel: the N-type channels have been identified. These channels have properties which are intermediate between those of N-and L-channels (Tsien et.al., 1988).

Calcium mobility can also be mediated by sodium/hydrogen exchange, as mentioned previously. This exchange is believed to be electrogenic, exchanging one calcium ion for three sodium ions. This means that hyperpolarization should favour calcium exit and depolarization should favour calcium entry (Eisner and Lederer,1985). In rod outer segments, potassium may also be involved such that four sodium ions, one calcium ion and one potassium ion are transferred (Eisner and Lederer, 1985). It is not known if this stoicheometry is unique to photoreceptor cells.

Calcium release from intracellular stores is mediated through the inositol trisphosphate receptor (InsP3-Receptor). This receptor was first purified from Purkinje cells (Supttapone et.al., 1988), and was reconstituted into lipid vesicles (Ferris et.al., 1989)which are sensitive to inositol trisphosphate and other inositol lipids too.

The calcium signal in response to EGF has no contribution from intracellular reserves and is due solely to calcium entry through VOCs (Moolenaar et.al., 1986 (a)and (b))-it still stimulates inositol phosphate turnover and DAG formation though.

It is believed that the calcium channel which is actiavted upon EGF binding to EGF-R is phosphtidic acid (Sawyer and Cohen, 1981). This is an anionic phospholipid which is formed from DAG in EGF treated cells that may be a calcium ionophore in model systems (Serhan et.al.,1982).

The absolute requirement for external calcium in EGF induced responses may reflect a calcium dependent coupling of individual EGF-Rs to the inositol phosphate generating system (Moolenaar et.al., 1986 (a) and (b)).

The observation that EGF caused an elevation in intracellular calcium levels was made initially in A431 cells (Moolenaar et.al.,1986 (a) and (b)). EGF caused the uptake of labelled calcium and the metabolism of inositol phosphates. This occurred in a calcium dependent way via the activation of voltage independent plasma membrane channels (Hill et.al.,1988).

The calcium response could be blocked entirely by treating the cells with TPA (Moolenar et.al., 1986 (a) and (b)). This suggests that protein kinase C is involved in the attenuation of

EGF induced calcium mobilization at some stage. However, such data is in conflict with that produced by Mozhayeva's group (Mozhayeva et.al.,1989).

It is now recognised that calcium from internal stores (i.e. that calcium mobilized by Ins 1,4,5 Ptd3) is involved in the EGF dependent calcium influxes. This was demonstrated by the stimulation of A431 cells with EGF (and also bradykinin and histamines). This treatment caused an elevation of the rate of formation of Ins 1,4,5 Ptd3 which is correlated with a rise in intracellular calcium in the absence of extracellular calcium influx (Hepler et.al.,1987).

There is some evidence in favour of the former hypothesis; such as that which shows EGTA treatment results in EGF induced intracellular calcium fluxes remaining unchanged (Johnson and Garrison, 1987). The model which is currently accepted by most is that in non-secretory cell at least, intracellular calcium levels show a biphasic response to growth factor stimualtion (Berridge, 1987). Phase one requires inositol trisphsphate dependent mobilization of calcium from intracellular stores and phase two, a prolonged phase, requires the inflow of extracellular calcium.

EGF and TGF-beta have an additive, enhancing effect on intracellular calcium release, suggesting that they could be acting on different calcium pools and/or channels (Muldoon et.al.,1988). This has been further hinted at by experiments which show that Actinomycin D has no effect on the EGF dependent influx of calcium but inhibits totally that part of it for which TGF-beta is responsible.

Such finding suggested to the authors that these growth factors may activate transcription of separate set of genes which culminate in the opening of calcium channels.

In the case of Swiss 3T3 fibroblasts, EGF and bradykinin cause enhanced intracellular calcium levels and also increase the rate of DNA synthesis (Olsen et.al.,1988). For these effects, extracellular calcium is not needed.

It would therefore seem that the situation is very complex, with different second messenger systems in acting in the presence of and/or in the absence of extracellular calcium to promote the entry of calcium into the cell and the subsequent stimulation of DNA synthesis (Hill et al.,1988 show that DNA synthesis induced by EGF is dependent upon the presence of extracellular calcium). How the calcium level effects are connected and to what extent they are regulated by other EGF induced effects is not known.

5.12. EGF-R and G-Proteins.

5.12.1.Introduction

G-proteins are a family of guanine nuleotide binding proteins (Gilman1987), which allow the coupling of signal transduction pathways of many receptors (Johnson and Dhanasekaran,1989). They are membrane bound proteins which comprise a transducing unit consisting of three parts: a receptor, a G-protein and an effector(s).

A classical G-protein is a member of a larger GTP binding protein family which comprises proteins such as translation factors, Ras, Rho and YPT and SEC4 of yeast (Gilman,1987). So far, eight mammalian G-proteins have been identified biochemically, and they transduce specific siganls (Gilman 1987). Some examples of receptors which are controlled by G-proteins are receptors for beta adrenergic agonist type hormones for instance adrenalin and neurotransmitters such as rhodopsin. G-protein controlled effectors include adenylyl cyclase, retinal cGMP-specific phosphodiesterase and phospholipase C (Gilman, 1987).

In molecular terms, G-proteins are composed of three subunits: alpha, beta and gamma. Alpha subunits are the heaviest and define individual G-proteins (Gilman,1987). Beta and gamma subunits are probably shared between different types of G-protein. (For a review on small GTPases see Hall, 1992)

Activity of G-proteins is achieved through the asssociation of GTP with the alpha subunit and by hydrolysis of this GTP to GDP plus Pi (which initiates the deactivation process) and finally by the dissociation of GDP from the G-protein.

G-proteins are thought to sort and amplify transmembrane signals. Specificity is achieved because each G-protein determines the flow of information from a specific number of receptors to a similar number of effectors. Sorting occurs in two steps, firstly activation of G-nucleotide exchange.Here the G-protein selectively interacts with the appropriate activated receptor only. The second step happens when the GTP-bound or active form of the G-protein binds to and activates the correct effector. The alpha chains contain the determinants for the correct sorting of these signals.

G- proteins amplify transmembrane signals through the use of GTP hydrolysis. This event acts as a timer to amplify signals. For example in the case of adenylyl cyclase, (Gilman,1987)

one hormone receptor complex is active for about one second and generates one or more GTP bound alpha chains which remain active for ten seconds.

5.12.2.The Involvement of G-Proteins in EGF-R Signal Transduction.

The preceeding sections serve to outline and emphasise the importance of G-proteins in the transduction of cellular signals. When the specific example of signal transduction induced by the binding of a ligand to the EGF-R is considered, the situation is a lot less clear.

A great deal of evidence states that there is no G-protein involvement in EGF-R signal transduction, yet reserch by an increasing number of groups contradicts this point of view by providing what they consider to be proof that a G-protein of some kind is involved in this process. The section which follows reviews the evidence for both hypotheses.

The cell line MD-468 (Pathak et.al., 1982) is a human breast cancer cell line with elevated numbers of EGF-R receptors: some 1.5-10-6 per cell (Filmus et.al., 1985). At high concentrations of EGF, growth is inhibited. This is not the case for some clonal variants of this cell line which have lost the amplified

egf-r: the MDA-468-S4 cell line. This variant has an absolute dependence on the addition of exogenous EGF for it's growth in anchorage independent condition.

This different response to EGF under the above noted growth conditions allowed the investigation of G-protein involvement in EGF-R signal transduction. At optimal EGF concentrations, different concentrations of pertussis toxin were added to MDA-468 and MDA-468-S4 cells. In both cell lines, an EGF concentration was reached where a concentration dependent reversal of EGF mediated growth inhibition was observed (Church and Buick,1989).

It was also found that in the case of the MDA-468 line, the EGF inhibition effect could be blocked by pertussis toxin while in the MDA-468-S4 cell line pertussis toxin had no effect.

Plasma membranes isolated from each cell line were subjected to ADP-ribosylation in vitro by [alpha-32P]-NAD in the presence of pertussis toxin. Plasma membranes from cells grown with and without EGF could incorporate 32P. This led to the labelling of a 41 K.D. polypeptide and other high molecular weight substrates. If pertussis toxin is omitted though, such an effect is no longer observed (Church and Buick,1989).

Plasma membranes from cells grown with pertussis toxin showed no ADP-ribosylation of the 41 K.D. polypeptide. This suggested to the authors that the ADP-ribosylation of this protein may be involved in the inhibitory effects of pertussis toxin on EGF mediated events in the two cell lines.

It therefore appears that in the MDA-468 cell line, growth cannot be inhibited by pertussis toxin alone in the presence of low EGF concentration. A G-protein pertussis toxin insensitive G-protein does not therefore seem to be involved. At high EGF concentrations though, which inhibits growth of the cells, post-binding events seem to be G-protein mediated and are blocked by pertussis toxin ADP-ribosylation of a putative G-protein.

For cells of the MDA-368-S4 cell line, cell proliferation under anchorage-independent conditions a pertussis toxin sensitive G-protein seems to be required. Pertussis toxin does not, however, prevent the increase in EGF dependent expression of c-myc and c-fos which suggests that EGF initiates two separate signal transduction pathways, one of which involves a G-protein (Church and Buick,1989).

The ability of known GTP binding proteins to act as phosphosubstrates for EGF-R has also been studied and a 22-25 K.D. high affinity G-protein was found to be a good

phosphosubstrate (Hart et.al.,1990). Phosphorylation of this protein is enhanced three fold by the addition of EGF. When it is incorporated into lipid vesicles without EGF-R, the protein is not phosphorylated and boiling of the protein abolishes its ablility to be phosphorylated. In addition, phosphoamino acid analysis reveals that this polypeptide is phosphorylated only on tyrosine residues.

The phosphorylation of the protein thus identified can be inhibited by GTP ys, GTP and GDP implying that the G-nucleotide occupied state of the protein inhibits it's phosphorylation by EGF-R. That is to say that activation of the G-protein may lead to its uncoupling from the EGF-R. Heterotrimeric G-proteins are thought to cause the release of these transducers from their receptors. The inhibition due to G-nucleotide occupation is probably a more general effect.

The fact that the nucleotide alters the conformation of the protein is possibly the feature responsible for its reduced phosphorylation status (Hart et.al.,1990).

The consequence of such an event could be that EGF-R can no longer effectively couple the GDP bound form of the 22 K.D protein in vitro or catalyze GDP-DTP exchange. A helper protein might therefore be needed to carry out this coupling to EGF-R, the process usually carried out by the beta/gamma complex (Fung,1983).

The 22 K.D. polypeptide described above is not a substrate for botulinum toxin under conditions in which it is ADP-ribosylated, the protein therefore cannot be Rho. Rap-1 is not phosphorylated by EGF-R, so the protein cannot be Rap-1 (Hart et.al.,1990). Finally, it does not cross-react with antibodies raised against Ras (Hart et.al., 1990).

Nakagawa showed that a mechanism involving a G-protein activated adenylyl cyclase may function in rat parotid gland acinar cells (Nakagawa et.al.,1991) i.e. that the cAMP signalling pathway activated by EGF can be mediated by a cholera toxin sensitive G-protein. That EGF has such an effect in one system may mean that it could do the same in others too. There is, however is great danger in extrapolating between possibly quite different systems.

Some other evidence suggesting the possibility of the involvement of a G-protein in EGF dependent signalling is as follows. In hepatocytes, pertussis toxin blocks the ability of EGF to stimulate inositol trishophate production (Johnson et.al., 1988 (a) and (b)). Pertussis toxin can block EGF inhibition of acid secretion in rat parietal cells (Atwell and Hansen, 1988).

In rat hepatocytes, inositol 1,4,5 Ptd3 accumulation is prevented by treatment with pertussis toxin (Johnson and Garrison,1987), as is phospholipase C activation by EGF (Johnson

and Garrison,1987). The inner medullary collecting tubule cell phospholipase A2 is activated by EGF and this process in inhibited by pertussis toxin (Teitelbaum, 1990 (a) and (b)). EGF stimulated accumulation of Ins 1,4,5 Ptd3 in WB cells is, in addition, abolished by pertussis toxin pretreatment of the cells (Johnson and Garrison,1987).

On the other hand, in A431 cells, by contrast, pertussis toxin has no effect on EGF-R dependent effects such as inositol phosphate metabolism (Pikes and Eakes, 1987). EGF-R has, in addition, no amino acid homology with any known member of the guanine nucleotide binding family of proteins (Carpenter,1987 and O'Dowd et.al.,1989).

It seems most likely that pertussis toxin is blocking EGF dependent effects upon phospholipase C activation in a cell type specific manner (Milligan, 1988). That is to say that different cell lines have different EGF induced signal transduction pathways (Teitelbaum, 1990 and Teitelbaum et.al., 1990 (a) and (b); Johnson et.al.,1988 (a) and (b) and Besterman et.al., 1984, 1985 and 1988). This could be brought about by the coupling of the EGF-R to different effector systems or by the activation of different phospholipase C isozymes.

Alternatively, EGF-R phopshorylation of components of G-proteins could take place which affects the way they interact with the other parts of the transducing unit (O'Brien et.al,1987; Krupinski et.al.1988; Katada et.al.,1985 and Crouch and Lapetina,1988).

Chapter Six: EGF-R AND BREAST CANCER.

6.1 Introduction.

EGF-R and other growth factor recptors have been found to be associated with the development of human cancers. Reviewed in this chapter are the ways in which EGF-R is thought to be involved in the genesis of breast cancer. Some other cancers will be discussed briefly, as will two related aspects of EGF-R/breast cancer research: the possible uses of EGF-R as a prognostic/diagnostic indicator and as a therapeutic target.

V-erb-B is the transforming gene of the Avian Eryhtroblastosis Virus (Downward et.al, 1984). In 1984 it was discovered that v-erb-B is highly homologous to the human egf-r gene and that it is derived from the cellular egf-r gene via a number of truncations and point mutations (Downward et.al.,1984). Both egf-r and erb-B genes code for proteins which have a built in tyrosine kinase activity.

6.2. EGF-R and Human Cancers.

The most frequent EGF-R anomaly in human cancers is overexpression of an apparently normal EGF-R protein. This is the case in squamous carcinomas, breast cancers, liver cancers, bladder cancers, pancreatic cancers, cancers of the respiratory tract and brain tumours. It can also be observed in cells lines such as A431, SCC-15 and MDA-468.

EGF-R over-expression is most commonly the result of an increase in the rate of transcription of the gene or is due to certain post-transcriptional mechanisms. Gene amplification is also a significant cause. The importance of EGF-R over expression in carcinogenesis is demonstrated by the fact that in KB cells full length anti-sense EGF-R mRNA can totally block egf-r expression. As a result of this treatment, the severity of the transformed phenotype is reduced (Moran et.al., 1988).

It has been proposed that the consequent enhanced tyrosine kinase activity is responsible for tumorignesis. Expression of high levels of EGF-R in NIH 3T3 cells has been found to induce a fully transformed phenotype in vitro that requires both functional c-erb-B expression and the presence of EGF in the growth medium (Di Fiore et.al., 1987 and Velu et.al., 1989).

Some cancers with very high levels of EGF-R (usually the result of gene amplification) are growth inhibited by high ligand concentrations, but are stimulated by low ligand concentrations. It has been suggested that the egf-r gene ampification is a reflection of genomic instability and of the selection of cells over-expressing EGF-R and so posessing increased growth potential in a limiting concentration of the ligand (Carlin et.al., 1989 and Gill et.al.,1985.).

In summary then, tumours of squamous origin and a number of cell lines can over-express EGF-R at their surface. Analysis by Southern blotting of the receptor gene shows amplification is observed frequently in certain squamous lines-but infrequently in others.

Some mutated EGF-R is also known. In A-431 cells, there is a truncated varient of EGF-R which has only the extracellular domain. It binds EGF, and is secreted (Ullrich et.al., 1984). Certain brain tumours have EGF-R which posesses short deletions in the ligand binding domain (Carpenter,1987). Such egf-r can be over-expressed too and has an increased tyrosine kinase activity in the absence of ligand (Carpenter,1987).

6.3.EGF-R and Cell Transformation.

The transforming potential of EGF-R is demonstrated in the following examples. Firstly, EGF-R N- and C- truncations occur in the Avian Eryhtroblastosis Virus. Such deletions of the v-erb-B gene leads to the loss of the extracellular domain and one of the autophosphorylation sites. There is also a point mutation in v-erb-B. The fact that there is an N-terminal deletion means that the resultant protein has no ligand binding domain and so cannot be down-regulated or internalized as the

normal receptor is. The theory is that the transforming ability may result from the constitutive activation of this protein's tyrosine kinase activity.

Autocrine stimulation of tumour growth has also been put forward to explain the transforming ability of growth factors and their receptors. Autocrine stimulation of cellular transformation correlates with TGF-alpha production by tumours which also over-express EGF-R (Derynck et.al.,1987).

6.4.EGF-R levels in Human Breast Cancer.

A number of methods have been used to assess the number of EGF-Rs on various human tumours, such as ligand binding assays, immunocytochemistry, immunohistochemistry, radioimmunoassay, Southern blotting, Western bloting, Northern blotting, P.C.R. analyses and E.L.I.S.A.

The first time that elevated numbers of EGF-Rs were demostrated on human tumours was by a group studying lung tumours using antibodies against EGF-R (Hendler and Ozanna, 1984). That increased EGF-R levels are actually involved in tumorigenesis is revealed by the finding that if the receptor's numbers are increased and activated ligand is present, fibroblasts (in vitro) are transformed (Di Fiore et.al., 1987).

Increased numbers of EGF-R can in the main be due to either increased transcription of egf-r or amplification of egf-r.

A summary table of the published literature on how EGF-R levels are associated with different human tumours is shown in Appendix Two.

6.5. EGF-R and Tumour Size, Stage, Grade and Spread.

Sainsbury's group have found that large tumour size, advanced stage, grade and spread of primary breast tumours is associated with EGF-R status. High levels of EGF-R were found to be associated with poorly differentiated tumours, large sized tumours and involvement of lymph nodes (Sainsbury et.al.,1985 (a) and (b)).

Macias reported that levels of EGF-R positivity are higher in human breast cancer metastatic deposits than in the primary tumours themselves (Macias et.al.,1986). The authors concluded that metastasis may involve the clonal selection of EGF-R positive cells which have a high metastatic potential. EGF-R alone is not the only requirement for metastasis though since approximately 50% of the secondary tumours lacked EGF-R. The only safe conclusion that might be drawn is that the primary tumour is not a homogeneous population of cells.

It has in addition been shown that metastatic deposits of human breast cancers are EGF-R positive far more frequently than primary tumours. For example, in 1 study, 25% of primary and 48% of secondary breast tumours are EGF-R positive (Skoog et.al.,1986). Again the data suggest that clonal selection is in action and that cells are being selected which have high EGF-R levels.

EGF-R levels are also positively correlated with accelerated growth rate in node-positive breast cancer (Spitzer et.al., 1988). This implies that for such patients, breast cancer growth is dependent upon EGF enhanced cell division.

6.6.EGF-R and Breast Cancer Sub-Groups.

A number of different sub-groups of breast cancer can be histologcally identified. The relationshp between the EGF-R status of a tumour and it's histological type was carried out in a retrospective study (Skoog et.al.,1986). The conclusions were reached:

Histological Sub-Type	EGF-R Detectable over the Total Number of Tumours Studied
Ductal	8/22
Lobular	0/9
Medullar	2/2
Colloid	0/4
Fibroadenoma	0/3

(Skoog et.al.,1986).

Other investigations carried out along similar lines to that above have revealed that, overall, some 40% of the breast tumours studied had detectable EGF-R (Perez et.al.,1984 and Fitzpatrick et.al.,1984 (a) and (b)). Ductal tumours compose about 80-85%of all cases (Perez et.al.,1984).

Some tumours were found to have no EGF-Rs. This may be because of a phenomenon of the transformed state, or because the tumour is exposed to exogenous EGF/TGF-alpha which causes EGF-R down-regulation (Heldin et.al., 1982). This possible restriction of EGF-R to certain tumours is important if EGF-R is used as a prognostic indicator.

The conclusion is that high EGF-R levels are associated with more advanced and aggressive breast tumours and so is an indicator of poor prognosis and early recurrence. Another investigation by the same group also indicates that increasing numbers of EGF-R on primary breast tumours is asssociated with increased metastatic potential (Sainsbury et.al.,1985 (a) and (b)).

Spitzer demonstrated that an enhanced level of EGF binding was correlated with lymph node involvement (Spitzer et.al.,1988). This finding has been confirmed in a number of studies such as those by Mori (Mori etal.,1991); Battaglia (Battaglia et.al.,1985 and 1989) and Toi (Toi et.al.,1989). There is, however, some controversy as to the correlation between EGF-R levels and the numbers of lymph nodes affected (Toi etal.,1989).

6.7. Interactions Between EGF-R and Hormone Receptors in Breast Cancer Development.

EGF-R and oestrogen receptors are believed to be inversely related such that 2 sub-groups of oestrogen receptor positive primary breast tumours can be identified (Harris,1989). These tumours can be either EGF-R positive (and oestrogen receptor positive) or EGF-R negative (and oestrogen receptor

positive).This phenomenon has in addition to having been shown immunohistochemically, has been demonstrated by ligand-binding too (Harris A.L.,1989).

Other reports confirming the inverse relationship between ER and EGF-R status are provided in studies by Koenders (Koenders et.al.,1991); Davidson (Davidson et.al.,1987); Bauknecht (Bauchnecht et.al.,1989) and Wrbra (Wrbra et.al.,1988).

Among the other hormone receptors which are thought to be involved in the process of mammary carcinogenesis are the prolactin receptor (Shiu, 1979); growth hormone receptor (Murphy et.al.,1984); insulin receptor (Benson and Holdaway ,1981) and somatostatin receptors (Reubi et.al.,1989 and 1990). The research into the molecular workings of these receptors in breast cancer development is not well advanced at present.

6.8. The Use of EGF-R as a Prognostic Indicator in Breast Cancer.

Evaluating the prognosis for any individual patient presenting with breast cancer (and any other disease too)is a very complicated matter. A number of interrelated prognostic

indicators are used to allow the clinician to make an assessment of the tumour in question, thus facilitating optimization of the therapeutic regime.

Contesso categorises such prognostic features as Clinical Factors and Pathological Factors (Contesso et.al., 1984). Clinical factors include factors such as age and reproductive history of the patient, the periods of time between discovery and diagnosis of the lesion, diagnosis and therapy and therapy and the appearance and site of the first recurrence or metastasis. Features of the tumour itself such as its size, TEM status, skin invasion and lymph adenopathy are also important prognostic features.

Contesso describes the patholgical factors as the size of the excised tumour, its histological grade and stage, lymph node involvement and the presence of necrotic areas within the tumour.

Such indexes allow the clinician to neither under treat nor over treat a patient. To select therapy and identify specific groups of patients which can receive more appropriate therapy, more and improved prognostic indicators (and response indicators) are sought. Among the most useful have proven to be hormone and growth factor receptors (Some oncogenes have also been shown to have a use in patient prognosis. For reviews see

Varley et.al., 1987, Perren, 1991, Wright et.al., 1989, Paterson et.al., 1991, Borg et.al., 1990 and Anbazhagan et.al., 1991).

Oestrogen receptor status is probably the most widely used of these indexes. The importance of oestrogen receptors is well established in that as well as allowing the cell to respond to various first line endocrine therapies, it seems that if such tumours should replase then these secondary tumours are more likely to respond to endocrine therapies.

Progesterone receptors have also been used as prognostic indicators. There are many reasons for its use in this way, with the fact that oestrogen causes progesterone receptor synthesis (Darnell et.al., 1986) being among the most important. In addition, as previously stated, the expression of progesterone receptors is believed to indicate that oestrogen receptors are functionally active (Horwitz and McGuire, 1978).

Considering EGF-R as a prognostic indicator, the following points can be made. Firstly, as has been already mentioned, oestrogen receptor and EGF-R are inversely related (Sainsbury et.al., 1985 (a) and (b) and Toi et.al., 1989). EGF-R is associated with less differentiated tumours (Sainsbury et.al., 1985 (b)), large tumour size (Sainsbury et.al., 1985 (a)), poor prognosis (Sainsbury et.al., 1987) and the involvement of lymph

nodes (Battaglia et.al., 1988). Those with the worst outlook are patients bearing EGF-R(+)/oestrogen receptor(-) tumours (Grimaux et.al.,1989).

Expression of EGF-R may also be associated with increased risk of early recurrence and death (Sainsbury et.al., 1987,and 1888 Toi et.al., 1990, Harris et.al., 1989 and Nicholson et.al., 1989). In one study involving two hundred and thirty one patients with unaffected axillary nodes, EGF-R was found to be the best prognostic indicator (Nicholson et.al., 1991). In node positive patients, EGF-R status was significantly correlated with oestrogen receptor status and histological grade allowing identification of sub-groups of patients (Grimaux et.al., 1989).

A study where EGF-R status of three hundred and three human breast cancers was assessed by ligand binding assay and revealed that fifty percent of the samples were EGF-R(+) (Bella et.al., 1992). These authors also noted that EGF-R expression was reduced in the more differentiated tumours.

Certain necrotic areas within breast tumours were found to express EGF-Rs (Reubi and Torhust, 1988). Such a finding complicates the use of EGF-R as a prognostic indicator and urges a restrained and considered approach to their use for such purposes.

It is hoped that EGF-R itself will in time become a therapeutic target for the poor prognosis group; for example by the creation of antibodies to the receptor; peptide analogues to EGF or TGF-alpha or drugs which are conjugated to EGF or TGF-alpha.

6.9.EGF-R and Breast Cancer Therapies.

Although a major killer of females in the prime of their lives, the death rate form breast cancer has remained virtually unchanged this century. As was previously discussed this is because of a lack of knowledge of the nature of the processes underlying the disease.

Usually, the concentrations of anti-tumour drug that will eradicate the tumour is lethal or at best be highly toxic to that patient. Amagase's group found that using EGF with certain anti-cancer drugs such as 5-fluorouracil in tumour cells which are rich in EGF-Rs causes the agent's cytotoxic effect to be enhanced towards the tumour cell without increasing toxicity (Amagase et.al.,1989).

Tumours can also develop cytotoxic drug resistance, and this is a very serious problem. EGF seems to have some ability to enhance the effacy of Doxorubicin in a clonogenic assay,

particularly in hormone-responsive patients (Hug et.al., 1986) and this could be a way to overcome this problem of multi-drug resistance. How EGF has this effect is not clear but it may be acting by increasing the passage of the cells through the cell cycle so rescuing them from Go and making them targets for the anti-cancer agents.

Another study was performed in the KB.C1 cell line: a human epidermal carcinoma cell line derived from KB cells via selection with Colchicine (Akiyama et.al., 1985). The findings have suggested that EGF-R may have a central role in drug-resistance.

KB.C1. cells were more resistant to EGF and anti-cancer agents than the original KB cells, and the authors found that this phenomenon is correlated with a reduction in the number of EGF-Rs (Takano et.al., 1989). A revertant clone: C1-R2 was isolated and had recovered EGF sensitivity and had enhanced EGF-R levels when compared to KB.C1.

In addition, the multi-drug resistance gene mdr-1 is expressd at high levels in KB.C1. cells and not at all in KB and C1-R2 cells. Multi-drug resistant cells are also less tumorigenic in nude mice (Takano et.al., 1989). The conclusion was therefore reached that reduced EGF-R expression is a phenomenon of multi-drug resistant cells and may even be the reason for reduced

tumorigenicity as noted above. For example, multi-drug resistance could cause altered expression of EGF-R, perhaps via chromosome 7 alterations.

The observations that EGF-R plays an important role in the prognosis and diagnosis of cancers as well as in their genesis, has suggested that it may be possible to use the EGF-R as a way to deliver cytotoxic drugs to a tumour. Targeting such very potent drugs specifically to a tumour, leaving surrounding normal cells undamaged would be a considerable step forward. In addition it could allow the clinicians to use more cytotoxic drugs and at a higher dose to treat the patient.

EGF has been fused to various fragments of the Diptheria toxin forming constructs called DAB 468 and DAB 389. EGF-R phosphorylates DAB 389 (Shaw et.al., 1991).

Both the fusion toxins have been shown to bind to EGF-R specifically and inhibit protein synthesis in human cancer cell lines, DAB 389 was the most cytotoxic though. This is due to the ADP-ribosylation of EF-2, i.e. the equivalent reaction to that which the Diptheria toxin carries out. DAB 389/EGF is at present in trials in nude mice bearing EGF-R expressing xenografts.

Using a similar reasoning, monoclonal antibodies against EGF-R have been used to try to prevent or reduce EGF-R acivity (i.e. signal transduction) in tumours over-expressing EGF-R. Antibodies against part of EGF-R have also been used to dissect it's mechanism of action (Kris et.al., 1985).

In the MDA-468 human breast cancer cell line, anti-EGF-R antibodies inhibit the EGF/TGF-alpha autocrine growth proliferation system in the absence of exogenous EGF (Ennis et.al., 1989). This implies that the antibodies are acting to block access of an exogenously produced ligand to EGF-R. Although a therapeutic application is not envisaged by the authors, it illustrates how such antibodies could be used to this end and suggests other possible uses such as in tumour diagnosis. Two monoclonal antibodies against the COOH- area of EGF-R have recently been evaluated as having potential diagnostic use (Troalen et.al., 1991).

In the Scid mouse model (scid/scid), established human M24 metastatic melanoma cells metastasize rapidly, in 100% of the mice (Mueller et.al., 1991). A monoclonal antibody against EGF-R specifically reduces this metastasis. Primary tumour growth is not altered though. It therefore seems that blocking

EGF/EGF-R binding has an anti-metastatic effect. This has also been observed in A431 cells, where an anti-EGF-R antibody has the same effect (Masui et.al.,1984).

Monoclonal antibodies have also been fused to various cytotoxic agents (the principle here is the same as that for EGF-R-cytotoxic drug fusion proteins). A nude mouse xenograft study showed that Vinca-EGF-R conjugates caused regression of established tumours (Gutowski et.al., 1991). Although at present untested, these results imply that pursuing such lines of investigation may prove to be useful, providing new therapeutic approaches to breast cancer.

Chapter Seven: CONCLUSION.

Chapter 7:Conclusion.

The literature concerning EGF-R activity is very large-this is particularly true in the case of EGF-R's role in human carcinogenesis. This thesis therefore limits itself to an appraisal of only certain core issues such as the structure (at the DNA and protein levels) of the receptor, how structure relates to function, what the various functions of the EGF-R are, how EGF-R activity might be regulated and how all this relates to the problem of breast cancer.

Breast cancer is a major killer of females world wide, and incidence is increasing every year-yet much progress remains to be made in terms of tackling the disease more effectively. There have been some recent improvements in the treatment and survival of hormone sensitive breast cancers, but the prospect for those with hormone insensitive breast cancer remains poor.

The reason for the disappointing statistic is that there is a serious lack of knowlege about the molecular processes which underly the process of carcinogenesis. In fact it might be more accurate to say that the real lack of knowledge concerns the processes of normal cell proliferation.

The first parts of this thesis therefore, deal with the basics of cancer biology and breast cancer biology. The main points to emerge from this study are that these processes have a very significant genetical component. Much evidence exists which seems to show that loss of certain areas of the genome and/or activation of specific oncogenes is associated with breast cancer development.

The crucial problem of cancer metastasis is also tackled with the observation that a discrete gene: nm23 is involved in this process. The significance of this finding is that identification of a gene opens the way to potential intervention, at least in theory, but in reality such knowledge is just one vital clue to be used and built upon by investigators.

Structural features of EGF-R are very important in another respect: they determine, define even the functions and activities of the receptor. This crucial area is reviewed in some depth in the thesis firstly because of its obvious significance and intrinsic interest.

Another reason for examining this subject in detail is that modern molecular biologial techniques have meant that new approaches to studying structure/function relationships have been used. EGF-R is a very good example, with processes like domain-swaps and site-directed mutagenesis in use towards a real and practical end.

Finally, EGF-R structure deserves close attention because herein lies the basis of EGF-R signal transduction. This process of transfer of information from the cell's environment inside the cell and its culmination in alteration in the pattern of gene expression is on which is at the heart of cellular activity.

Although here we are concerned with these processes as they pertain to carcinogenesis, very important general rules are learned about the processs itself. These rules are often applicable universally (maybe in a slightly modified manner).

Then the cellular effects of EGF-R activation on inositol phosphate metabolism and ionic fluxes are reviewed as is the possible involvement of a G-protein in these processes. It was noted that these areas: EGF-R as a protein kinase and modulator of intracellular activities are very controversial. This issue of how EGF-R tyrosine kinase activity is activated is, in particular a highly contentious one with two main theories both with much strong supporting and conflicting evidence.

It is believed that the intracellular signalling processes are peturbed in cancerous cells. Again exactly what form this may take is not known but many theories exist. This is discussed in direct relation to human breast cancer development in this thesis, with the autocrine theory of cellular proliferation being the most widely held.

As well as looking at a number of ways in which alterations in EGF-R signal transduction may be involved in breast cancer development genetical aspects of breast cancer as considered too. Their importance has been touched on previously and I feel that their usefulness (i.e. in terms of allowing a basic understanding of the carcinogenic process and allowing cancer to be tackled clinically) will only increase in the future.

Perhaps it could be considered as an indirect involvement in breast cancer, but a very important aspect of EGF-R in this process is its use as a prognostic (or even diagnostic)indicator. In this way clinicians can identify specific patients and treat them more appropriately. An aid with improves patient treatment and/or the usefulness of a therapy must be welcomed. In fact far from being a side-line issue, it is probably fair to say that the use of EGF-R as a prognostic indicator is at present the major contribution of EGF-R research to oncology.

EGF-R biology holds great promise for the future. As was previously stated, advances in technology have meant a better understanding of EGF-R signal transduction is now possible. Such advances, when they come will have significance not solely in the field of EGF-R signal transduction, but in the wider area of cellular signalling too.

Work is also being done which sees the EGF-R as a potential for anti-cancer therapy. Again cancer will not be the only disease to benefit from such advances since any pathology involving EGF-R could be tacked in this way, psoriasis for example.

Conjugation of various cytotoxic agents to parts of EGF hijacks and takes advantage of the fact that EGF binds solely and specifically to EGF-R. In this way it is hoped that the abberant EGF-R activity can be controlled if not abolished totally. Finally, certain compounds which have been designed to inhibit kinase activity are now available.

This research is now so advanced that agents which specifically inhibit the tyrosine kinase activity of EGF-R are available. The long-term aim is that such compounds will form the basis of a new generation of drugs which will be active specificically against tumours/cells containing elevated amounts of EGF-R.

Such work is still in it's infancy and there is a long way to go with many problems to be overcome. However, it amply shows the importance of EGF-R and its contribution to many areas of biological science and oncology and highlights how well placed it is to continue to be important and contribute in the future.

APPENDICES.

Appendix One : Protein Kinase Inhibitors.

Name	Reference
Epiderstatin	Osada et.al.,1989; Sonoda et.al., 1991.
Erbstatin	Takekura et.al., 1991.
Flavonoid Derivatives	Cushman et.al., 1991.
Genistein	Watanabe et.al., 1989 and 1991; Linassier et.al., 1990 and Honda et.al., 1991.
Lavendustin	Chen et.al., 1987; Glenny et.al., 1987 (a) and (b) and Hsu et.al.,1991.
Phosphonate Containing Derivatives	Burke et.al., 1991.
ST 638	Watanabe et.al., 1989 and 1991.
Sulphonylbenzoyl-nitrostyroles	Wacker et.al., 1990.
Tyrphostins	Gazit et.al., 1989 and 1991; Yaish et.al., 1988; Levitzki et.al., 1990 and 1991; Coussens et.al., 1985 Lyall et.al., 1989 and Posner et.al., 1989.

Appendix Two:EGF-R Levels in Human Tumours.

SITE OF TUMOUR	COMMENT UPON EGF-R LEVELS	REFERENCES
Brain:Astrocytoma, Glioma, Medulloblastoma, Oligodenroglioma, Meningioma,and Glioblastoma mutiforme.	High levels of EGF-R.The gene is often amplified. No evidence of mutation of the gene. EGF and/orTGF-alpha levels are often elevated too. Common in Astrocytes and gliomas and could be an aid to diagnosis.	Tuzi et.al.,1991; Wang et.al.,1987; Reubi et.al., 1989; Arita et.al., 1989; Agosti et.al., 1992; Hurtt et.al., 1992 and Torp et.al., 1991.
Head and Neck	Increased levels of EGF-R are commonly observed in squamous carcinomas.10-20% have an amplified egf-r gene. May be an aid to diagnosis.	Gullick,1991
Mouth:Oral mucosa,floor of the mouth and tongue.	High levels in squamous cell carcinomas and severe forms of leukoplakia. Some gene amplification for diseses involving the floor of the mouth and the tongue.	Couwenhoven et.al., 1990 and Shirasuna et.al., 1991
Oesophagus	Two types of squamous cell carcinomas with either high or low EGF-R levels. In about 20% of cases gene is found to be amplified, and this is associated with high disease potential.	Ozawa et.al., 1989 and Mydlo et.al.,1989;

Larynx	In cases of severe dysplasis or carcinoma in situ, EGF-R is present in increased amounts, but not in less aggressive forms. It may therefore be an aid to diagnosis. In basal and parabasal cells of the mucosa EGF-R can indicated advanced disease. May be correlated with seroid hormone receptor levels too.	Miyaguchi et.al. 1991; Scambia et.al. 1991(a) and (b).
Thyroid	High and low affinity groups of EGF-R are seen in thyroid carcinomas: undifferentiated, squamous and papillary types. In advanced forms of the disese it is the high affinity type that is increased. In adenomas and ademomatous goitres, the EGF-R numberswere in the normal range. In Grave's Disease receptor Kd values are reduced as are overall numbers of receptors. Papillary carcinomas have increased TGF-alpha and EGF genes overexpressed too, as are their mRNA 's, suggesting an autocrine loop mechanism is in action. There are reduced levels in Grave's Disease thyroid cells and in addition, these EGF-R have lowered binding affinty.	Kanamori et.al. 1989; Miyamoto et.al., 1989.

Kidney	Increased levels of TGF-alpha and EGF-R are noted in stage1 clear cell carcinoma. No mRNA increases can be detected. Actual levels depend on the precise positon in the nephron: they are highest in the proximal tublues and lowest in the distal convoluted tubules, i.e. in the inner medullary region.	Mydlo et.al.,1989.
Bladder	Primary transitional carcinoma has elevated levels of EGF-R, and this is associated with a high stage and poor prognosis. Also, the invasive but not the superficial tumour has a high EGF-R content. This may allow interaction with urinary EGF which is present at a high level. Gene rearrangement is rare but enhanced mRNA content is very common as is an increase in p53 levels.	Messing et.al., 1990; Berger et.al., 1988; Wood et.al., 1992; and Wright et.al., 1991.

Prostate	Well differeniaed tumours have the most EGF-R's. Benign hyperplastic prostate conditions have the highest levels of all, but precise numbers varied greatly. Highest levels of EGF-R are observed in benign neoplastic conditions, and in cancerous states, actual levels of EGF-R expression vary depending on the histological grade of the tumour with well differentiated tumours having highest EGF-R expression.	Maddy et.al. 1989; Maygerden et.al., 1992; MacDonald and Habib, 1992; Lubriano et.al., 1992 and Maddy et.al., 1989.
Placenta	The chorionic villi trophoblast is a epithilium around a mesenchymal core. In the first trimester of pregnancy, is is composed of a number of layers formimg the villous cytotrophoblast and acellular syncytiotrophoblast. The former structures have very high levels of EGF-R's. They proliferate to form exravilous cytotrophoblast cell columns which anchor the placenta into the maternal decidua and uterus and have very low EGF-R levels. Tyrosine kinase activity is reduced too and changes in the glycosylation pattern are noted which suggests that at different stages of pregnancy, EGF-R is likely to have different molecular weights.	Tavare and Holmes,1989 and Bissonnette et.al., 1992.

Colo-Rectum

Normal mucosa and muscularis mucosa has very high EGF-R levels. The receptor is present at very low levels in the circular and longitudunal muscles surrounding the gut. It is unusual for carcimonas and adenomas to have elevated EGF-R levels, and it is believed the observed increase has to do with tumour progression and not initiation. Some 25% of carcimonas were metastased and were poorly differentiated suggesting that the presence of EGF-Rs is correlated with poor prognosis. Tyrosine kinase activity is reduced as the disease progresses too. Ligand binding assay has shown EGF-R levels are higher in the proximal region of the colon compared to the distal region-but only in healthy colon and not cancerous states.	Zimmerman et.al 1988; Sakanoue et.al., 1991; Koretz et.al. 1990 Magnusson et.al. 1989 and Koenders et.al. 1992.

Skin	Epithelial skin tumours, especially squamous cell carcinomas have a slight increase in EGF-R levels. EGF-R levels are elevated in actively growing seborrheic keratoses and acrochordons (skin tags), but not in their non-growing versions. Viral dependent lesions have varying levels of EGF-R expression but Verruca vulgaris has none.	Bauknecht et.al. 1985; Nanney et.al 1992; Nazmi et.al. 1990 and Gan, and Hollenberg, 1989.
Coronary Arteries	Sites of EGF binding seem to be concentrated in the tunica media, with highest concentration at the boundaries between this region and the tunica adventitia. One study has also shown there are no high affinity EGF-R or increased EGF-R mRNA production in this tissue.	Gan and Hollenberg, 1989, Damstrup et.al., 1992.
Lung	A 1-3 fold increse in EGF-Rlevels is seen in ung tumours.In neonates, EGF-R is abnormally increased or present where normal foetuses have none and is indictive of respiratory tract damage. This	Hwang et.al.,1986; Stahlamn et.al., 1989; Dazzi et.al.,1989; Veale et.al.,1987; Tateishi et.al.,1990 Gullick,1991;

	is visualised as acute and chronic lung disease in the epithelium of the airways implying that wounding may cause an elevation of EGF levels which in turn causes an increase in either numbers or activity or both of EGF-R s. There is little or no over expression of the receptors seen in small cell lung cancer, 84% or so squamous cell carcinomas and 56% of adenocarcinomas do have elevated EGF-R numbers. High EGF-R staining is seen in large cell tumours too. Such increased levels of EGF-R are associated with poor prognosis.	Cerny et.al, 1986 and Dittadi et.al., 1991.
Liver	Elevated numbers of EGF-R are seem on the plasma membranes of cells derived from hepatoblastomas, and hepatocelluar carcinomas, when compared to normal or inflammed tissues. Partial hepatectomy results in a 2 fold increse in the level of EGF-R mRNA and protein levels within about 4 hours; reaching the nadir 36-48 hours later.	Fukusato et.al.,1990; Johansson and Andersson1990; Francavilla et.al.,1991

Stomach	Gastric carcinomas, as they advance, show elevation in the number of EGF-R's:4% of early and 34% latetumours show this. TGF-alpha is at a high level in most tumours too, but mRNA levels remain at normal levels. Increased EGF-R's have often a mutated egf-r or have the gene present in mutiple copies.	Sugiyama et.al., 1989; Yoshida et.al.,1990; and Lemione et.al., 1991.
Uterus	EGF-R semms to be correlated with histological grade, depth of myometrial invasion, oestrogen and progesterone receptor status, metastasis or high rate of recurrence. Elevated levels of EGF-R expression are very common in cancers of the uterus. There are no fluctuations in EGF-R levels throughout the menstrual cycle.	Berchuck et.al., 1989 (a) and (b); Bauknecht et.al., 1989; Battaglia et.al., 1989 and Prentice et.al., 1992.
Vulva	Invasive squamous carcinomas have very high levels of EGF-R expression but without rearrangement of egf-r.	Battaglia et.al., 1989 and Gullick et.al., 1985.

Cervix	Very high levels of EGF-R expression in cervical cancers often with mRNA levels elevated too. Stromal cells remain EGF-R negative. Associated with poor prognosis. Increased levels in dysplatic states too, with the level of dysplasia correlated with expression of EGF-R mRNA byond the mid-epithelium.	Goppinger et.al., 1989, Berchuck et.al., 1989, Battaglia et.al., 1989; Kohler et.al., 1989 and Mittal et.al., 1990 and 1992 ;
Ovaries	In serous type ovarian adenocarcinomas some 74% of cases have increased EGF-R levels, but the mucinous conditions have enhanced EGF-R in around only 17% of cases. Such EGF-R expression is associated with poor prognosis.	Baucnecht et.al., 1989 and Gullick, 1985.

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